MOLECULAR MECHANISMS OF ANTIMICROBIAL RESISTANCE AND POPULATION DYNAMICS OF *NEISSERIA GONORRHOEAE* IN SASKATCHEWAN (2003-2011)

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ABSTRACT

Gonorrhea is caused by the human pathogen *Neisseria gonorrhoeae*. More than 106 million new cases of *N. gonorrhoeae* infections occur each year worldwide. There is no vaccine available against gonococcal infections and treatment of gonorrhea with antibiotics is the only way to eradicate infection. The high prevalence of antibiotic resistance (AMR) in this microorganism makes the effective treatment of gonococcal infections increasingly problematic. The emergence of AMR, especially to extended spectrum cephalosporins (i.e. cefixime and ceftriaxone) which are the last possibilities for single dose treatment options for gonococcal infections, is a serious concern. Gonorrhea may become an untreatable infection in the near future.

Saskatchewan (SK) has one of the highest rates of gonorrhea in Canada. In order to better characterize the gonorrhea epidemic in SK, the objectives of the present research were to determine the prevalence and trends of AMR and emerging AMR mechanisms in *N. gonorrhoeae* isolates. AMR mechanisms were ascertained for the first time in SK in order to identify genetic causes of resistance. This was completed by determining and analyzing the DNA sequences of various genes - *penA*, *mtrR*, *porB ponA, gyrA, parC mtrR*, 23S rRNA alleles and *erm* –implicated in gonococcal AMR. The population dynamics of the *N. gonorrhoeae* isolates in SK was investigated by DNA based molecular methods to determine strain distribution, evolution of AMR phenotypes, and association between strain types (STs) and AMR genotypes and phenotypes.

N. gonorrhoeae isolates (n=427) from Saskatchewan (2003-2011) were susceptible to antibiotics now recommended for treatment - cefixime, ceftriaxone and spectinomycin. Over 95% of the isolates tested were also susceptible to penicillin (96%) and ciprofloxacin (95.5%), antibiotics no longer recommended for treatment, and azithromycin (99.4%). Tetracycline resistance was also high (50.1%).

N. gonorrhoeae isolates that were resistant to the antibiotics tested and also those isolates with MICs ≥0.003 mg/L to cefixime and ceftriaxone were analyzed (n=146) to determine their

resistance mechanisms. This analysis revealed that reduced susceptibility to ceftriaxone and cefixime and resistance to penicillin is mediated by specific mutations in penicillin binding protein 2 (PBP2), in the promoter and dimerization domains of MtrR and porin protein (PorB). Novel mutations and combinations of mutations were noted. Ciprofloxacin resistant *N. gonorrhoeae* isolates carried double mutations in GyrA (S91F and D95G/N) and a S87R or S88P substitution in ParC. Isolates resistant to azithromycin had specific mutations in all the four alleles of 23S rRNA as well as in the DNA binding domain of MtrR. Most resistance was chromosomally mediated while plasmid-mediated resistance to penicillin (0.93% of penicillin resistant isolates) and tetracycline (3.3%) was low.

DNA based strain typing methods such as *porB*-DNA sequencing, *N. gonorrhoeae* multi-antigen sequence typing (NG-MAST) and multilocus sequence typing (MLST) showed that the gonococcal population in SK differs appreciably from both other Canadian provinces and from strains reported internationally. MLST analysis, which ascertains the evolution of isolates over time, demonstrated that penicillin and tetracycline resistant isolates in SK evolved through spontaneous mutations in established lineages. Ciprofloxacin and azithromycin resistant *N. gonorrhoeae* isolates, on the other hand, were introduced into SK from outside the province. Significant associations between particular mutation pattern combinations in resistance determining genes and specific NG-MAST STs were identified e.g. NG-MAST ST 25 was associated with specific combined mutation patterns in PBP2, MtrR and PorB and antibiotic susceptibility; and, NG-MAST ST 3654 was associated with another PBP2/MtrR/PorB mutation pattern, chromosomal resistance to penicillin and tetracycline and elevated MICs to cefixime.

This research shows the importance of regional antimicrobial susceptibility monitoring. In the context of SK, this means that local surveillance of gonococcal AMR may be used to develop policies for regional treatment guidelines which promote the prudent use of antimicrobials for treatment, including those antibiotics which may no longer be used in other regions due to higher AMR rates. Further, the significant association between particular AMR mutation pattern combinations and specific STs indicates that AMR might be predicted. These results should assist in the development of non-culture-based tests for the diagnosis of

gonococcal AMR similar to nucleic acid amplification tests used to diagnose *N. gonorrhoeae* infections.

Key words: *Neisseria gonorrhoeae*, antimicrobial susceptibility/resistance, molecular determinants of antimicrobial resistance, molecular epidemiology, molecular typing, strain types

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Thank you all and best wishes,

Sidharath

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LIST OF ABBREVIATIONS

A	Auxotyping
AA	Amino acid
AFLP	Amplified fragment length polymorphism
AMR	Antimicrobial resistance
AP-PCR	Arbitrarily primed polymerase chain reaction
ARDRA	Amplified ribosomal DNA gene restriction analysis
ASGP-R	Asialoglycoprotein receptor
ATP	Adenosine triphosphate
AUH	Arginine-hypoxanthine-uracil-requiring phenotype
bp	base pair
CCR-5	Chemokine receptor type 5
CDC	Centers for Disease Control and Prevention, the United States
CEACAM	Carcinoembryonic antigen-related family of cell adhesion molecules
CFU	Colony forming unit
CLSI	Clinical and Laboratory Standards Institute
CMP	Cytidine monophosphate
CMPR	Chromosomally mediated penicillin resistance
CMR	Chromosomally-mediated resistance
CMTR	Chromosomally mediated tetracycline resistance
CR3	Complement receptor 3
DGI	Disseminated gonococcal infection
DNA	Deoxyribonucleic acid
ESSTI	European Surveillance of Sexually Transmitted infections
EUCAST	European Committee on Antimicrobial Susceptibility Testing
GASP	Gonococcal Antimicrobial Susceptibility Surveillance Program
GCMBK	Gonococcal medium base with 1% Kellogg's supplement
GISP	Gonococcal Isolate Surveillance Project (the United States)
GRASP	Gonococcal Resistance to Antimicrobials Surveillance Programme
GyrA	DNA gyrase

hCG	Human chorionic gonadotropin
HIV	Human immunodeficiency virus
HPA	Health Protection Agency (the United Kingdom)
HPSG	Heparin sulfate proteoglycans
HTH	Helix–turn–helix motif
ID	Index of discrimination
LAMP	Lysosome associated membrane protein
LAMP	Lysosome associated membrane protein
LHr	Lutropin receptor
LNnT	Lacto-*N*-neotetraose
LOS	Lipo-oligosaccharide
LPS	Lipopolysacchride
MAb	Monoclonal antibody
MDa	Megadalton
MIC	Minimal inhibitory concentration
MLEE	Multiple locus enzyme electrophoresis
MLST	Multilocus sequence typing
MLVA	Multilocus variable-number tandem repeat analysis
MSM	Men who have sex with men
MtrR	Multiple transferable resistance repressor
NAAT	Nucleic acid amplification test
NG-MAST	*Neisseria gonorrhoeae* multi-antigen sequence typing
NR	Nutrient non-requiring phenotype
nt	Nucleotide
Opa	Opacity-associated protein
P	Proline requiring
P^+	Piliated
P^-	Nonpiliated
ParC	Topoisomerase IV
PBP	Penicillin binding protein
PCR	Polymerase chain reaction

PCU	Proline-citrulline-uracil-requiring phenotype
Pen^I	Penicillin intermediately susceptible
Pen^R	Penicillin resistant
Pip	Proline iminopeptidase
PFGE	Pulsed field gel electrophoresis
PHAC	Public Health Agency of Canada
PI	Porin protein I
PIA	Proin protein I allele A
PIB	Porin protein I allele B
PID	Pelvic inflammatory disease
PMN	Polymorphonuclear leukocyte
PorB	Porin protein B
PPNG	Penicillinase-producing *Neisseria gonorrhoeae*
PUH	Proline-uracil-hypoxanthine requiring phenotype
QRDR	Quinolone resistance-determining region
QRNG	Quinolone resistant *Neisseria gonorrhoeae*
RAPD	Randomly amplified polymorphic deoxyribonucleic acid
RE	Restriction endonucleases
RFLP	Restriction fragment length polymorphism
RNA	Ribonucleic acid
rRNA	Ribosomal ribonucleic acid
S	Serotyping
SDCL	Saskatchewan disease control laboratory
SK	Saskatchewan
SLV	Single locus variance
SSuN	STD Surveillance Network
ST	Strain type
STD	Sexually transmitted diseases
STI	Sexually transmitted infections
TbpB	Transferrin binding protein B
TRNG	Plasmid-mediated tetracycline resistant *Neisseria gonorrhoeae*

VNTR	Variable number tandem repeat
WHO	World Health Organization
WPR	Western Pacific regions

CHAPTER ONE

INTRODUCTION

1.1 Epidemiology and Clinical Features of Gonorrhea

1.1.1 Prevalence of Gonorrhea Worldwide

Neisseria gonorrhoeae, a Gram-negative coccus, is an obligatory human pathogen causing gonorrhea, which is one of the most common sexually transmitted infections (STIs) worldwide. More than 106 million new cases of gonorrhea are reported annually (WHO 2012). Gonorrhea remains an underreported disease worldwide often because of unrecognized infections, inadequate laboratory diagnosis facilities, poor reporting systems and reluctance on the part of the patients or physicians to report the disease to public health authorities (Tapsall, 2009). Lack of knowledge about STI prevention in populations at increased risk of the disease, as well as in the general population, also contributes to the high prevalence of gonorrhea and other STIs (Cohen et al 2000).

Gonorrhea has been a notifiable disease in Canada since 1924 and is the second most commonly reported bacterial STI (PHAC 2010a). From 1991 to 1997, males and females experienced an overall decline in the reported rates of gonorrhea infection (Fig 1.1A). Between 1999 and 2008, reported rates of gonorrhea infection among males and females increased by 95.1% and 151.1%, respectively. Overall, the reported rate of infection was higher for males than for females. In Canada, gonorrhea prevalence rates have been rising since 1997 (Hansen et al 2003). In 2009, over 11,000 cases of gonorrhea were reported in Canada, a rate of 33.1 cases per 100,000 population, which is more than double the numbers for 1997 (14.9 cases/100,000, PHAC 2011a). This steady increase in rates since 2000 presents a significant public health concern in Canada. The majority of the reported gonorrhea cases were in the under 30 years of age group, in males and females between 15 to 24 years old in 2009 (PHAC 2011b).

Saskatchewan (SK) has one of the highest gonorrhea rates in all the provinces of Canada (PHAC 2011a). From 1991 to 1999, males and females experienced an overall decline in the reported rates of gonorrhea infection (Fig 1.1B). Between 2000 and 2008, reported rates of gonorrhea infection among males and females increased by 230% and 340%, respectively. Since 2000, the reported rate of infection was higher for females than for males. The gonorrhea rate in the province was 84.9 per 100,000 people in 2009, 2.5 times higher than the national rates

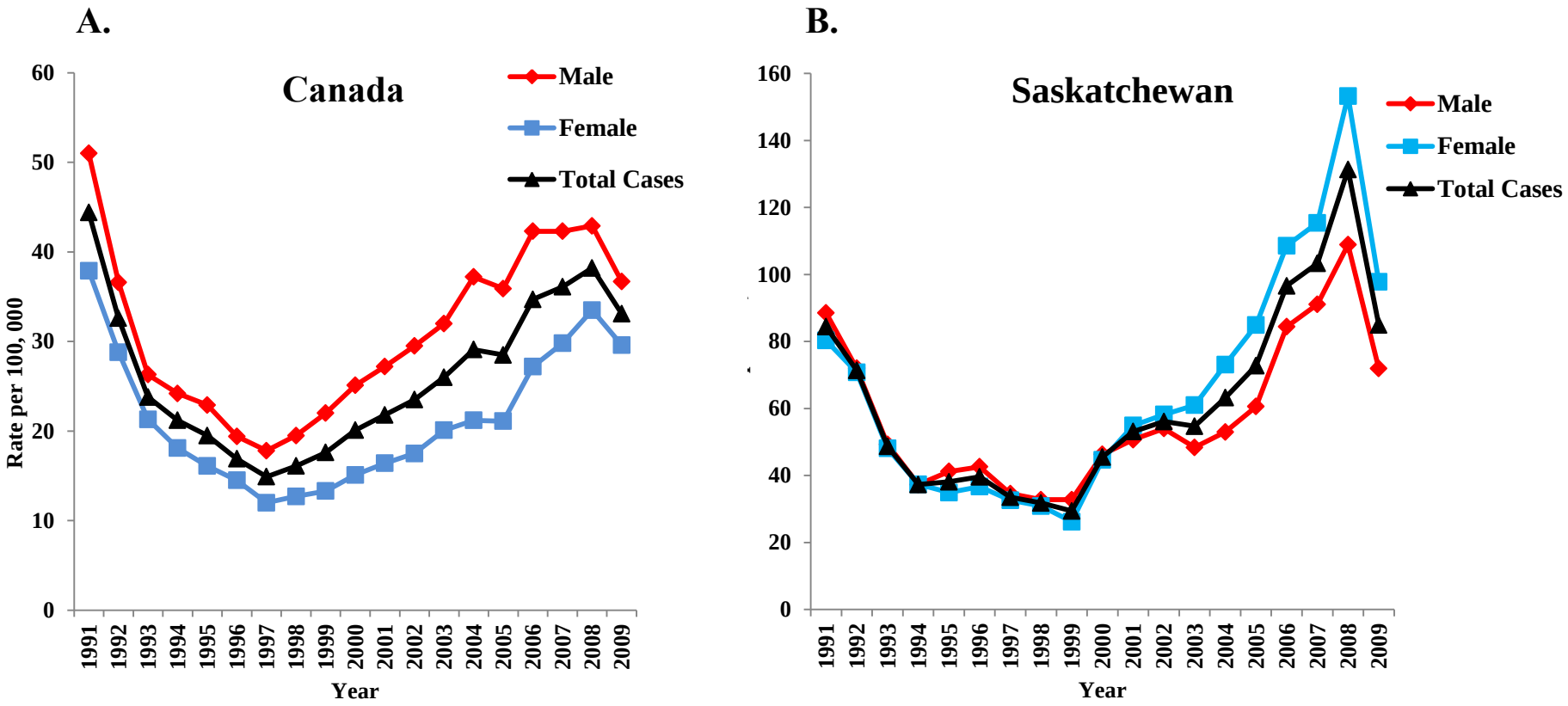

Fig. 1.1 Prevalence of gonorrhea in Canada and Saskatchewan

A. Gonorrhea rates in Canada

B. Number of reported gonorrhea cases in Saskatchewan.

Data were derived from the Public Health Agency of Canada (PHAC 2011a)

(PHAC 2011a). In 2009, the incidence rates of gonorrhea in SK among men and women were 71.9 and 97.8 per 100,000 population (PHAC, 2011a).

The increase in prevalence of gonorrhea and other STI's has been partly attributed to an increase in unprotected sex, particularly since HIV (Human Immunodeficiency Virus) infections are no longer considered as 100% fatal (PHAC, 2009). The use of noninvasive nucleic acid amplification tests (NAATs) has also increased the diagnostic efficiency for gonorrhea, resulting in higher levels of detection and reporting rates compared to rates using invasive specimen collection coupled with culture methods used in the past (Whiley et al 2006; Dillon 2011).

Gonorrhea is the second most commonly reported notifiable disease in the United States (CDC 2011). In 2010, a total of 309,341 cases of gonorrhea were reported in the United States with a rate of 100.8 cases per 100,000 population. The incidence rates of gonorrhea in the United States in 2010 among men and women were 94.1 and 106.5 per 100,000 population. According to the STD Surveillance Network (SSuN), 23.2% of the reported cases of gonorrhea in the United States were estimated to be in men who have sex with men (MSM; CDC 2011).

According to World Health Organization (WHO 2011) 87.65 million new cases of gonorrhea were reported worldwide in 2005 with an overall incidence of 25.6 per 1000 population. In females incidence rates were 23.38 per 1000 population with 39.91 million new cases in 2005, whereas in males the incidence estimates were 27.47 per 1000 population with 47.74 million new cases. The gonorrhea incidence estimates for the year 2005 were highest for African region (49.14 per 1000 population) and lowest for European region (10.12 per 1000 population) (Fig 1.2). The incidence of gonorrhea was higher in males compared to females in African region, region of Americas, South-East Asian region and East Mediterranean region. In the European and Western Pacific regions higher numbers of cases were recorded in females than males.

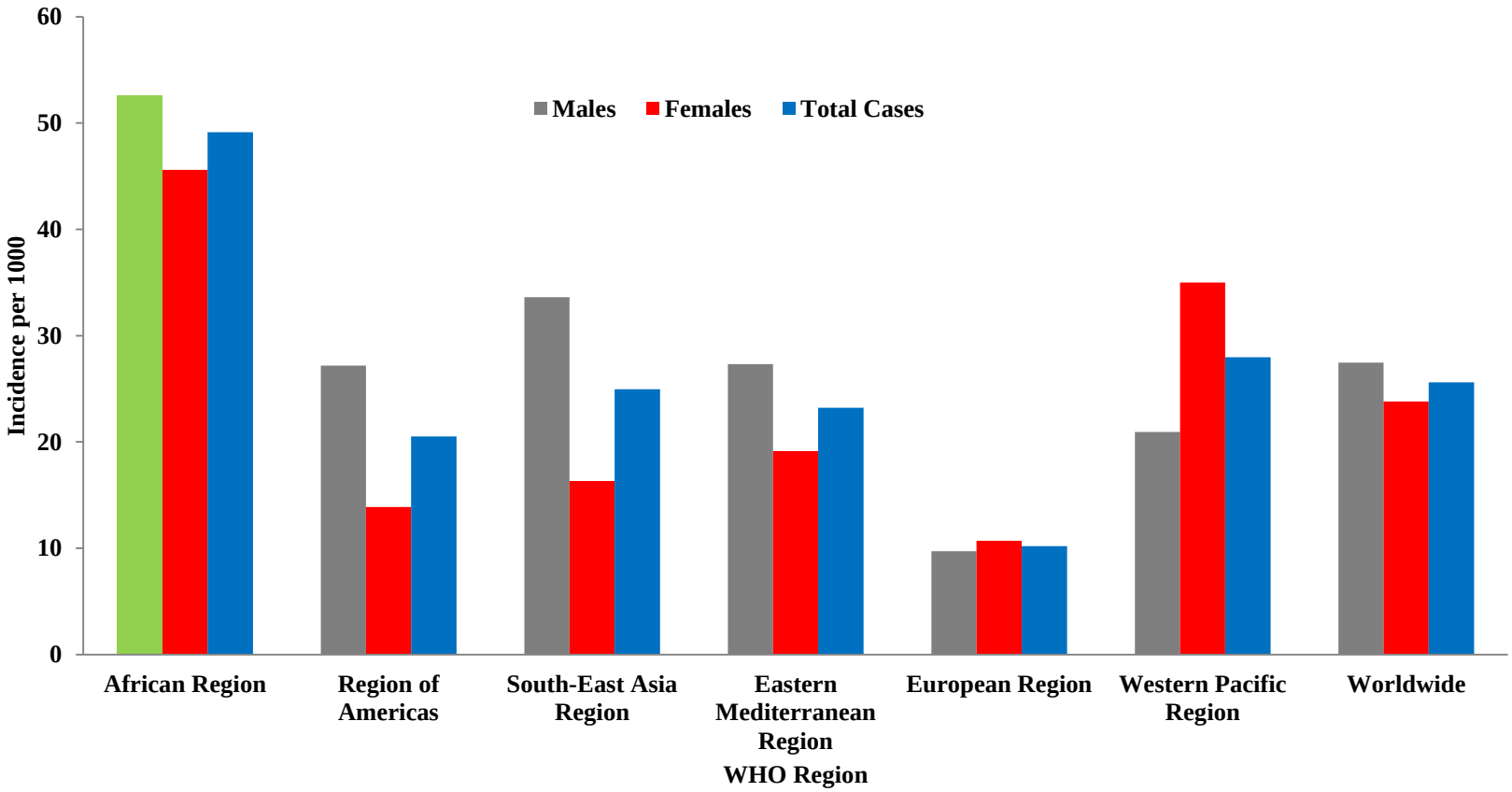

Fig. 1.2 Incidence of gonorrhea in different WHO regions in 2005.

Data were derived from the World Health Organization (WHO 2011).

1.1.2 History and Identification of *Neisseria gonorrhoeae*

Neisseria are cocci 0.6-1.0 µm in diameter. These occur singly but more often in pairs. The species *N. weaver*, *N. elongate* and the proposed new species, *N. baclliformis sp. nov.* are exceptions, often arranged as diplobacilli or in short chains (Elias et al 2011). The genus *Neisseria* includes at least 21 members (Knapp 1988). *N. gonorrhoeae* and *N. meningitidis* are obligatory human pathogens. Other members of the genus are non-pathogenic commensal species, although some of them can cause opportunistic infections in humans (i.e. *N. lactamica*). These non-pathogenic species are important because of their occasional diagnostic confusion with gonococci or meningococci (Sparling 2008). High rates of homologous recombination between *Neisseria* spp. result in the evolution of *Neisseria* spp. with mosaic alleles i.e. alleles generated in a recipient strain by recombination of regions of similar genes from closely related donor species. The genetic characters of these newly evolved species remain unclear (Hange et al 2005).

N. gonorrhoeae was the first discovered species of genus *Neisseria*. Gonococci were initially observed by Albert Ludwig Sigesmund Neisser in 1879. The organisms were demonstrated within polymorphonuclear leucocytes (PMNs) in pus smears from patients with gonorrhea. *Neisseria gonorrhoeae* was successfully cultivated by Bumm and by Lestikow and Loeffler in 1882 (Morse & Genco 1998).

N. gonorrhoeae is a Gram-negative diplococcus, which produces oxidase (Elias et al 2011). *N. gonorrhoeae* produces acid from carbohydrates by oxidation and utilizes glucose only (Morse and Genco 1998). It is a fastidious organism and grows on selective media under specific conditions. It requires an energy source such as glucose, pyruvate and lactate to grow. All *N. gonorrhoeae* strains require cysteine for the growth. Glutamine and cocarboxylase are required for the growth of the majority of strains during primary isolation. Because of these specific growth requirements, complex media are required for the primary cultivation of this organism (Morse and Genco 1998). Selective media used for *N. gonorrhoeae* cultivation includes the addition of antibiotics such as vancomycin, colistin and nystatain to prevent growth of commensal bacteria and yeast (Elias et al 2011).

Isolates are usually grown in nutrient rich media containing glucose, glutamine, thiamine, cysteine, cystine, adenine, vitamin B_{12}, p-aminobenzoic acid and ferric nitrate. *N. gonorrhoeae* inoculated media are incubated at 35^{o}C for 24-72 hr under humid conditions and in an environment containing 5-7% CO_2. The colonies of *N. gonorrhoeae* are approximately 1mm in diameter, opaque, glistening and convex. The colonies expressing pili and opacity proteins have well defined edges, whereas non-pliated colonies have a more diffused edge and are more glistening (Elias et al 2011). Gonococci occur in multiple colony types when grown on a clear agar medium and viewed under obliquely transmitted light. Small convex glistening colonies type 1 and type 2 colonies can be distinguished from larger flattened type 3 and type 4 colonies. Small colonies are known to be piliated (P^+) and large colonies are nonpiliated (P^-) (Kellogg et al 1963).

N. gonorrhoeae can be differentiated from other *Neisseria* species through enzymatic action on chromogenic substrates. The presence of the enzyme proline iminopeptidase (Pip) is specific to gonococci, Pip negative *N. gonorrhoeae* strains have also been identified (Tapsall 2007). The presence of such strains can result in false negative reactions. GonoCheck II and *Neisseria* PET are the two commercially available kits for identifying and differentiating *N. gonorrhoeae* on the basis of enzymatic reaction (Elias et al 2011).

Tests based on a combination of carbohydrate utilization tests and direct enzyme detection assays e.g. Api NH and RapID NH are available commercially for the rapid confirmation of *Neisseria* spp. The Api NH comprises four sugar utilization tests (glucose, fructose, maltose and sucrose), eight enzyme substrates and an acidimetric penicillinase test. The RapID NH has two carbohydrate utilization tests (glucose and sucrose), 10 enzyme tests and one resazurin reduction test (Elias et al 2011).

All immunological tests used for identification of *N. gonorrhoeae* detect outer membrane porin protein, PorB (PorB IA and PorB IB) of *N. gonorrhoeae* by a pool of monoclonal antibodies (Knapp et al 1984). The Phadebact Monoclonal GC test is a co-agglutination test (Carlson et al 1987; Dillon et al 1988). The test utilizes inactivated *Staphylococcus aureus* cells coated with antibodies bound to Fc portions through staphylococcal protein A. The Fab part of

the antibody is free to react with *N. gonorrhoeae*. GonoGen II is a colorimetric test in which antibodies are adsorbed on metal-sol particles and give red color as positive test (Dillon et al 1988). The Micro Trak Culture Confirmation Test uses fluorescein isothiocyanate labeled antibody for confirmation of *N. gonorrhoeae* (Dillon et al 1988). Positive specimens are identified as diplococci with apple green fluorescence (Elias et al 2011).

The non-culture *N. gonorrhoeae* diagnostic tests are based on detection of *N. gonorrhoeae* nucleic acids from non-invasive clinical specimens i.e. urine (Hook and Handsfield 2008). These tests provide rapid and sensitive detection of the *N. gonorrhoeae* and are in use since 1990s. The non-culture *N. gonorrhoeae* tests can be classified as hybridization assays and nucleic acid amplification tests (NAATs) (Whiley et al 2006).

NAATs for the detection of *N. gonorrhoeae* became available in the early 1990s. These assays use a specific oligonucleotide probe to hybridize directly to *N. gonorrhoeae* nucleic acid present within a specimen (Whiley et al 2006). NAATs have additional advantages of providing performance similar to that of endocervical swabs when voided urine or vaginal swabs are used as specimens (Whiley et al 2006; Hook and Handsfield 2008). This simplifies specimen collection and permits gonorrhea screening in settings where genital examination is not possible and disease prevalence is high. In addition, a single specimen collected for NAATs can be tested for both *N. gonorrhoeae* and *Chlamydia trachomatis* (Whiley et al 2006; Hook and Handsfield 2008). Amplicor CT/NG, ProbeTec and Gen-probe APTIMA are the commercially available NAATs. The test format includes amplification of target DNA from the specimen; the amplified DNA is hybridized to oligonucleotide to target specific probes and detection of the probe bound amplified DNA by colorimetric determination (Elias et al 2011).

1.1.3 Synopsis of Pathobiology of *N. gonorrhoeae*

A number of virulence factors have been identified that allow N. gonorrhoeaeto successfully adapt to variable microenvironments within its host. The steps involved in the pathogenesis of the gonococci are adherence to epithelial cells, internalization, invasion and dissemination (Sparling 2008). Multiple gonococcal surface molecules independently or collectively contribute to the mechanism involved in the adherence to human epithelial cells. Two well documented adherence ligands are pili and opacity associated (Opa) outer membrane proteins (Edwards and Apicella 2004; Sparling 2008).

Gonococci appear to be able to multiply and divide intracellularly and some gonococci can exit from the cell by exocytosis. The invasion by *N. gonorroheae* is dependent on expression of PorB, Opa proteins and lipooligosaccharide (LOS) (Edwards and Apicella 2004; Sparling 2008).

In addition to these adhesion and invasion factors, a number of gonococcal components undergo phase and antigenic variation, resulting in greater antigenic variability in the molecules on the surface of the cells during the course of the disease (Edwards and Apicella 2004; Edwards and Butler 2011). The major virulence factors involved in the pathogenesis of *N. gonorrhoeae* are briefly reviewed below.

Pili play an important role in initial attachment of the gonococci with host cells and also provide the ability to ascend mucosal surfaces by twitching. The exact mechanism of this twitching motility is not clear (Wall & Kaiser 1999). This twitching can be the result of a random alternation due to thermal motion, between hydrophilic surface interactions and hydrophobic surface interactions. It has also been suggested that twitching can be due to forceful retraction and extension of pili from one cell to another that will result in a twitch (Wall & Kaiser 1999). The I-domain of α_1 β_1 or α_2 β_1 integrin on epithelial cells of urethral mucosa and the I-domain of complement receptor 3 (CR3) in cervical mucosa act as receptors for the gonococcal pilus (Edwards et al 2002; Edwards & Apicella 2005). Pilus-host cell interaction causes calcium flux which results in exocytosis of host cell lysosomal contents and redistribution of lysosome-

associated membrane protein 1 (LAMP1) (Ayala et al 2001). LAMP1 is one of the proteins required for maturation of gonococci containing vacuoles and gonococcal killing inside the host cell (Binker et al 2007). Pilus attachment also causes rearrangement of the host cell cytoskeleton, facilitating entry of *N. gonorrhoeae* (Griffiss et al 1999).

Opa proteins are structurally and functionally related proteins encoded by 11 independently expressed *opa* genes (Bhat et al 1991). Opa proteins as represented by Opa_{50} and $Opa_{52.}$ Opa_{50} adheres to heparin sulfate proteoglycans (HPSG). Opa_{52} binds to the carcinoembryonic antigen-related family of cell adhesion molecules (CEACAMs or CD66) (Edwards & Apicella 2004; Edwards & Butler 2011). Opa_{50}-HPSG interaction releases diacylglycerol which activates spingomyelinase. Spingomyelinase produces ceramide from spingomyelin and ceramide causes cytoskeletal disruption of host cells (Grassme et al 1997). Opa-CAECAM interactions result in the activation of a series of tyrosine kinases resulting in cytoskeletal changes in host cells which facilitate the entry of gonococci (Hauck et al 1998). Opa proteins are not required for an interaction with epithelial cells but they do play a role in the interaction of gonococci with neutrophils (Rest et al 1982; Edwards & Butler 2011; Johnson & Criss 2011).

Neisseria carry LOS molecules in their outer membranes, which mimic mammalian cell sugar moieties called glycosphingolipids. LOS does not possess repeating the O-antigen sugar that comprises the polysaccharide side chain of lipopolysacchride (LPS) present on other Gram-negative bacteria. This structural similarity between LOS and mammalian glycosphingolipid protects the bacterium from the host-derived immune system (Edwards and Apicella 2004; Edwards & Butler 2011). LOS also serves as a site for the deposition of sialic acid. Deposition of sialic acid occurs through sialyltransferase using host cytidine monophosphate (CMP)-*N*-acetylneuraminic acid. The presence of sialic acid on gonococcal LOS also confers serum resistance to *N. gonorrhoeae* (Smith et al 1995). LOS is an important virulence factor in *N. gonorrhoeae* pathogenesis and binds asialoglycoprotein receptor (ASGP-R) on the urethral epithelium (Harvey et al 2001; Edwards & Butler 2011). Sialylation of LOS impairs LOS-ASGP-R binding (Harvey et al 2001; Shell et al 2002).

Gonococcal porins, as PorB are water-filled channels through which small molecules can cross the outer membrane. PorB serves as a secondary adhesion protein, binding to the I-domain of CR3 along with the pilus, resulting in a tighter gonococcus-receptor interaction (Edwards et al 2001; Edwards & Butler 2011). Gonococcal porin has the ability to translocate into eukaryotic cell membranes (Edwards & Apicella 2004). *N. gonorrhoeae* has been shown to induce apoptotic cell death in epithelial cells and phagocytes *in vitro* (Muller et al 1999). Cells treated with purified porin respond with an immediate, transient uptake of extracellular Ca^{2+} and cells start to form extensive membrane blebs. PorB isoform, (PIB) has been shown to increase the expression of the anti-apoptotic genes in male urethral epithelial cells, which aids survival of the bacterium in the protected intracellular environment (Binnicker et al 2003, 2004). Porin also causes cytoskeletal rearrangements through fragmentation and remodeling of actin filaments, which facilitates the entry of gonococci in the host cell and inhibits phagosome maturation (Wen et al 2000; Mosleh et al 1998).

Approximately 45% of women with gonococcal cervicitis develop an ascending infection (Edwards & Apicella 2004). Expression of CR3 decreases from the ectocervix to the fallopian tubes with the progressive increase in expression of the lutropin receptor (LHr) (Edwards et al, 2001). Adherence to the LHr by gonococcal ribosomal protein L12 is proposed to mediate gonococcus-fallopian tube interaction (Chen et al 1991; Spence et al 1997). Human chorionic gonadotropin (hCG) is a natural ligand for the LHr and can competitively inhibit the LHr-gonococcal interaction (Gorby et al 1991). Gonococcal-LHr interaction could possibly result in serious complications with increased risk of spontaneous abortions associated with *N. gonorrhoeae* infections (Edwards & Apicella 2004)

1.1.4 Control, Transmission, Clinical Manifestations and Complications of Gonorrhea

Gonorrhea control strategies include safe sex practices and early diagnosis followed by antibiotic treatment. Contact tracing and primary diagnosis on the basis of clinical symptoms and laboratory diagnosis are also important to check the spread of infection in a population. There is no vaccine available against *N. gonorrhoeae*, therefore, the prevention and treatment of gonorrhea relies primarily on early diagnosis and treatment with antibiotics. Important strategies for managing gonorrhea infections include preventing the misuse or inappropriate use of antibiotics, evaluation of appropriate treatment for gonorrhea, monitoring of antimicrobial use, as well as ensuring the supply of the right drugs in appropriate quantity at the right time and at the right dose (Tapsall et al 2009a).

Transmission: Patterns of STI transmission depend upon sexual partners, within sexual networks. A sexual network is a social network defined by the sexual inter-relationships within a defined group of people (Kretzschmar 2000). STI's are "network diseases" because the links established through sexual contact, and each contact is significant in defining a sexual network (Day et al 1998).

Gonorrhea transmission primarily occurs through small groups of high risk people, called "core groups" (Yorke et al 1978; Jolly & Wylie 2002). Core groups are joined directly or indirectly through sexual contact and transmit infection within the population through their sexual partners (Yorke et al 1978; Potterat et al 1985; Anderson & May 1992; Jolly & Wylie 2002). Bridging populations, the individuals in the lower-risk group act as a link between the core infected groups and the general population (Fig 1.3) (WHO 2007). Therefore, effective identification of core groups is critical for the proper and effective control of STIs (WHO 2007). It has been recommended that intervention strategies should focus on high-risk (core groups) and bridging populations and not the low risk general population (WHO 2007). Sexual networks appear densely connected through core groups in early epidemic phases and therefore, interventions focusing core groups should have a large population effect during epidemics (Ward 2007).

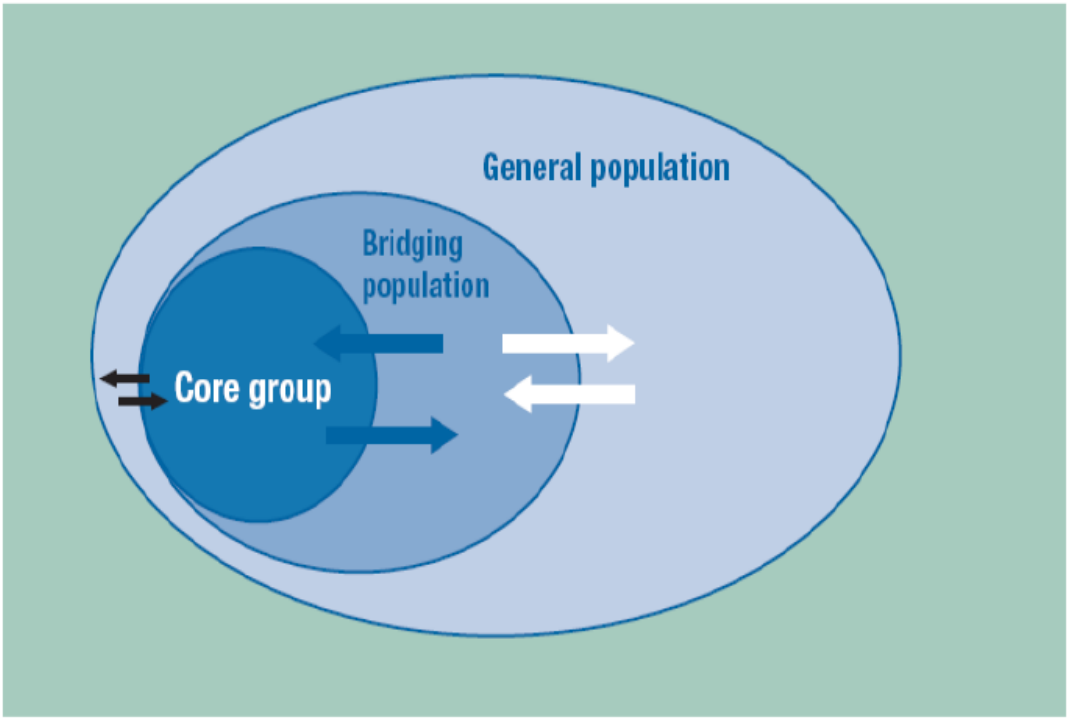

Fig. 1.3 Transmission dynamics of sexually transmitted infections at the population levels. Source: WHO 2007.

In endemic regions, interventions focusing on a larger proportion of people will have greater impact (Ward 2007).

A combination of epidemiological and molecular biological methods can work to establish a much better view of sexual networks in gonorrhea transmission (Day et al 1998), when contrasted to the traditional contact tracing methods for the identification of sexual networks in STI studies. The latter consist of STI surveillance and screening, identification of index cases, partner notification, and contact tracing through extended interviews. The local transmission networks of STI's can be identified by combining the molecular information of strain types (STs) with demographic data (Ward et al 2000; Choudhury et al 2006; Risley et al 2007).

Two types of transmission components within a large sexual network have been identified for STIs, radial and linear, (Wylie and Jolly 2000). Radial components are characterized by the presence of a central individual with a partner range of 5 to 13 people. In linear components, the partner range is 1 to 4 people with a larger number of distinct geographic locations. Linear components represent an important reservoir for gonorrhea maintenance and spread (Wylie and Jolly 2001). The linear component has extensive interconnections present within a large sexual network, resulting in frequent bridging events between different demographic groups (Wylie et al 2005).

Clinical Manifestations and Complications: The primary infection sites of *N. gonorrhoeae* are the mucous membranes lined with noncornified columnar or cuboidal epithelial cells of the urogenital tract (the urethra in males and the uterine cervix in females), the rectal mucosa, the conjunctiva and pharynx (Sparling 2008; Hook & Handsfield 2008). Over 50% of women and 10% of men who have gonorrhea are asymptomatic (Hook & Handsfield 2008). This consequently delays the treatment and facilitates the spread of the pathogen (Sparling 2008; Hook & Handsfield 2008).

In men, acute anterior urethritis is the most common manifestation of gonococcal infection. The incubation period of gonorrhea ranges between 1 to 14 days but the majority of

men develop symptoms within 2-5 days. Clinical disease is primarily manifested by urethral discharge or dysuria. Urethral discharge is initially scanty and mucopurlent in appearance and usually becomes frankly purulent and profuse within 24 hr of the onset of the infection. Patients complain of a urethral itch and pain or burning sensation when passing urine (Sparling 2008; Hook & Handsfield 2008).

In women, the endocervical canal is the primary site of urogenital gonococcal infection followed by the urethra. The incubation period of urogenital gonorrhea is variable in women but symptoms usually appear within 10 days of infection. The most common symptoms include increased vaginal discharge, dysuria and intermenstrual bleeding. There may be a strong smelling vaginal discharge which is often thick and yellow/green. Many infected women have cervical abnormalities such as mucopurulent cervical discharge, cervical erythema and edema and endocervical bleeding. Purulent discharge may also be expressed from urethra and periurethral gland or Bartholin's duct (Sparling 2008; Hook & Handsfield 2008).

Gonococci causing cervicitis may also spread to the rectal mucosa in women. The rectal mucosa is also a frequent site of infection in homosexual men. Rectal gonorrhea is usually asymptomatic in women and in MSM can be associated with overt proctitis. Other symptoms of rectal gonorrhea include anal erythema and edema, painless mucopurulent discharge, scant rectal bleeding, severe rectal pain, tenesmus, and constipation (Sparling 2008; Hook & Handsfield 2008).

Pharyngeal gonorrhea is transmitted to the pharynx by orogenital sexual contact and is acquired by fellatio (Hook & Handsfield 2008). Pharyngeal gonorrhea is common in MSM. Gonoccoccal infection can cause pharyngitis or tonsillitis but over 90% of pharyngeal infections are asymptomatic. The transmission of pharyngeal gonorrhea to sex partners is rare because of low bacterial loads in pharynx but can increase the risk of disseminated gonococcal infection (DGI) in individuals (Bissessor et al, 2011).

In men, ascending infection of *N. gonorrhoeae* causes epididymitis that can lead to infertility and acute or chronic prostatitis. Other complications in men include seminal vasiculitis and infections of Cowper's and Tyson's glands (Bolan et al 1999; Hook & Handsfield 2008).

In women, the most common consequence of untreated gonorrhea is pelvic inflammatory disease (PID), which includes salpingitis, endometritis, tubo-ovarian abscess or pelvic peritonitis (Hook & Handsfield 2008). PID causes scar tissue formation in the fallopian tubes. A fertilized egg may not be able to pass into the uterus through scarred fallopian tube. This may cause implantation of embryo in the tube leading to tubal (ectopic) pregnancy. In addition to ectopic pregnancies, tubal and peritubal damage after PID may result in chronic pain, miscarriage, gynecological morbidity and possibly death of the mother (Morse & Beck-Sague 1999; Paavonen et al 2008; Hook & Handsfield 2008). Apart from PID, Bartholin's gland abscess is the most common urogenital complication of gonorrhea in women (Hook & Handsfield 2008).

Untreated gonorrhea can spread through the bloodstream causing DGI. It is manifested by acute arthralgia, arthritis, tenosynovitis, dermatitis or a combination of all these findings resulting in joint pain and skin lesions as necrotic pustules. Patients with DGI can develop endocarditis with valvular damage or may suffer with meningitis (Sparling 2008; Hook & Handsfield 2008).

Gonococcal ophthalmia neonatorum, an infection in the eyes of the newborn, is acquired during passage through the birth canal of an infected mother. The initial conjunctivitis rapidly progresses and, if untreated, results in blindness due to corneal ulceration and perforation (Morse & Beck-Sague 1999; Kohlhoff and Hammerschlag, 2008).

Infection with *N. gonorrhoeae* has been associated with increased susceptibility to and enhanced incidence rates of infections with the sexually transmitted human immunodeficiency virus (HIV) (Galvin & Cohen 2004; Ding et al 2010). The findings of clinical trials and epidemiological studies has shown role of STIs, including gonorrhea, in HIV transmission (Wasserheit 1992; Galvin & Cohen 2004; Ding et al 2010). Gonorrhea enhances the infectiousness of HIV due to several pathophysiological factors including increased HIV viral

load in the urethra, semen, and cervical fluid due to a high degree of inflammation, as well as increased HIV replication due to the influx of polymorphonuclear cells (PMNs) during gonococcal infections (Galvin & Cohen 2004). Gonorrhea also makes a person more susceptible to HIV infection due to damage of the columnar epithelial barriers and increased infiltration of HIV co-receptor (CCR-5) containing cells (Wasserheit 1992; Galvin & Cohen 2004). In response to *N. gonorrhoeae* infection, genital epithelial cells produce cytokines, chemokines and defensins to modulate HIV infection and infectivity (Galvin & Cohen 2004; Jarvis & Chang et al 2012). *N. gonorrhoeae* infected women have more endocervical T cells and monocyte-derived dendritic cells providing more targets for HIV (Levine et al 1998; Zhang et al 2005). Antimicrobial peptides, such as human defensin 5 and 6, induced by gonococcal infection, also promote HIV infectivity (Klotman et al 2008). Infection with *N. gonorrhoeae* also enhances HIV-1 infection of primary resting CD4+T cells through TLR2 activation (Ding et al 2010). All these interactions are of great concern because the majority of HIV infections are sexually transmitted (Cohen 2004).

1.2 Surveillance of Antimicrobial Resistance in *Neisseria gonorrhoeae*

1.2.1 Antimicrobial Resistance in *N. gonorrhoeae*

N. gonorrhoeae has developed antimicrobial resistance (AMR) against most classes of antibiotics. According to the accepted definition of gonococcal treatment efficacy, a cure rate of over 95% is required for an antibiotic to be recommended for gonorrhea treatment (Handsfield et al 1992; WHO 1989, 2001; CDC 1987). Thus, an antibiotic should not be used in situations where resistance is observed in greater than 5% of gonococcal isolates tested (PHAC 2006; Newman et al 2007). AMR in *N. gonorrhoeae* is increasing in prevalence, and resistance to new classes of antibiotics has spread globally (Dillon 2011; Ohnishi et al, 2011a,b; Unemo et al, 2010, 2011, 2012a,b; Bolan et al, 2012).

N. gonorrhoeae strains have developed high levels of resistance against several antimicrobial agents including sulfonamide drugs (1940s), penicillin [chromosomal (associated with changes to existing and non-plasmid-borne genes), 1950s; plasmid mediated (resistance plasmid acquisition by bacteria), 1976], tetracycline [chromosomal, late1950s; plasmid

mediated, 1986] and quinolones (late 1990s) globally (Tapsall 2005; Stathi et al 2006; Wang et al 2006; Enders et al 2006; Bala et al 2007; Lewis 2010; Shafer et al 2010; Bolan et al 2012). This has resulted in the removal of these oral antimicrobials from gonorrhea treatment guidelines (Tapsall et al 2009a; Lewis 2009; Bala et al 2010; Dillon 2011).

After the worldwide development of quinolone resistant *N. gonorrhoeae* (QRNG), extended spectrum cephalosporins have been widely recommended for treating gonococcal infections and are considered the last single-dose bullet against the disease (HPA 2005; PHAC 2006; Japanese Society for sexually transmitted infections 2006; Wang and Zhang 2007; CDC 2010).

The recent reports of the emergence of *N. gonorrhoeae* strains with resistance or reduced susceptibility to extended spectrum cephalosporins (cefixime and ceftriaxone) and treatment failures with extended spectrum cephalosporins are a serious concern worldwide (Ameyama et al 2002; Ito et al 2004; Tapsall et al 2009a,b; Unemo et al 2010; Allen et al 2011, 2013; Ohnishi et al 2011a, b; Unemo et al 2011; Dillon 2011; Martin et al 2011, 2012; Bolan et al 2012). Small minimum inhibitory concentration (MIC) increases (i.e. MIC creep) to third generation cephalosporins has been widely reported from different parts of world (Chisholm et al 2010; Ross and Lewis 2011; Dillon 2011; Martin et al 2011). The WHO has a global alert on untreatable gonococcal infections (WHO 2012). It is speculated that emergence and subsequent spread of *N. gonorrhoeae* strains resistant to extended spectrum cephalosporins will render gonorrhea untreatable in future, at least with the single antibiotics in single doses, which has been the best practice for over half a century.

The incidence of antibiotic resistant *N. gonorrhoeae* has also increased in Canada (PHAC 2008a; Martin et al 2011). Gonococcal resistance to penicillin and tetracycline is long established in Canada (PHAC, 2008b). In Canada, tetracycline resistance was observed in the early 1970s (Dillon et al 1978a) and was highly prevalent in gonococcal isolates tested in the 2000s (Greco et al 2003). Penicillin and tetracycline resistance decreased from 14.2% and 23.3% in 2000 to 5.6% and 14.3% in 2003 in Canada and increased again after 2003 (Martin et al 2011; PHAC 2013). Similar trends were observed for erythromycin resistance (Martin et al 2011; PHAC 2013). The

prevalence of ciprofloxacin resistance in Canada increased from 1.3% of cultured isolates in 2000 to 36.0% in 2010 (Martin et al 2011; PHAC 2013). Azithromycin resistance has remained sporadic in Canada since 2000 (Martin et al 2011; PHAC 2013). *N. gonorrhoeae* isolates with reduced susceptibility to extended spectrum cephalosporins have been also been reported from Canada (Allen et al 2011; Martin et al 2012; PHAC 2013). A shift in the modal MICs of currently recommended antibiotics, azithromycin (0.25 mg/L in 2000 to >2.0 mg/L in 2011) and ceftriaxone (0.016 mg/L in 2000 to 0.063 mg/L in 2011), has been observed in Canada (Martin et al 2011; PHAC 2013).

1.2.2 Antimicrobial Resistance Mechanisms in *N. gonorrhoeae*

Over their long history of evolution, bacteria have developed various ways to protect themselves from antimicrobial agents. These mechanisms can be generally categorized into four groups (Jayaraman 2008, 2009): 1) restriction of intracellular drug concentration by influx and efflux, 2) chemical modifications or destruction of drugs, 3) alterations of drug targets in bacteria, and 4) bacterial tolerant states which are associated with persistence, biofilm formation, and swarming. Some mechanisms mediate cross-resistance to multiple unrelated drugs, whereas more than one resistance mechanism can co-exist in a microorganism against a particular antibiotic.

A systematic understanding of gonococcal AMR mechanisms is critical for the development of new and effective antimicrobial agents. Mechanisms of antibiotic resistance in *N. gonorrhoeae* include mutation of the antibiotic target, enzymatic breakdown of antibiotics, decreased permeability of the bacterial membrane and, the presence of active efflux systems (Fig 1.4) (Alekshun and Levy 2007; Shafer et al 2010). AMR in *N. gonorrhoeae* develops due to genetic changes, which can occur by spontaneous mutation, and or due to horizontal transfer (DNA uptake and homologous recombination) of the genetic material from other bacteria (Shafer et al 2010).

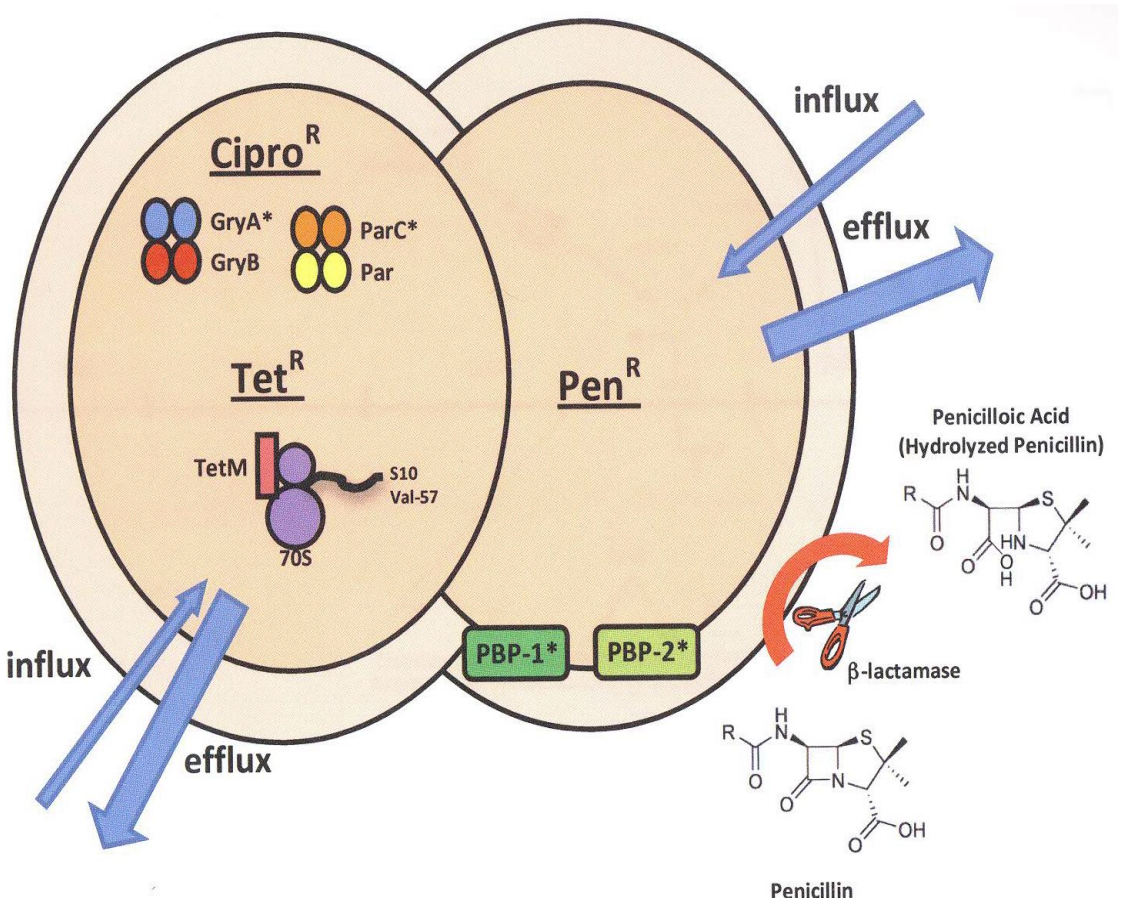

Fig. 1.4 Mechanisms of penicillin, tetracycline and ciprofloxacin resistance in *N. gonorrhoeae*.

Pen resistance (Pen^R) is due to actions of β-lactamase, decreased influx and increased efflux of the antibiotic and modifications of penicillin binding proteins (PBP), PBP 1 and PBP 2.Tetracycline resistance (Tet^R) is attributed to modifications in influx and efflux mechanisms. High level of Tet^R is due to attachment of TetM protein to ribosomes which prevents tetracycline binding to ribososmes. A substitution at Val-57 to Met in ribosomal protein S10 along with decreased influx and increased efflux of the antibiotic provides chromosomal tetracycline to *N. gonorrhoeae*. Ciprofloxacin resistance (Cip^R) is due mutations in DNA gyrase (GyrA) and topoisomerase IV (ParC).

Source: Shafer et al 2010.

The antimicrobial resistance determinants in *N. gonorrhoeae* can be both plasmid mediated and chromosomally mediated. Plasmid-mediated resistance may confer resistance to penicillin (i.e. penicillinase-producing *N. gonorrhoeae*, PPNG), or to tetracycline (i.e. tetracycline resistant *N. gonorrhoeae*, TRNG). Penicillinase production in PPNG is mediated by a TEM1-type β-lactamase encoded by the Tn*A* transposon Tn*2*, which is truncated and includes 84% of *tnpR*, noncoding sequences, the entire *bla* gene, and the right inverted repeat (IR-R) (Chen and Clowes, 1987; Dillon & Yeung 1989; Pagotto et al 2000). These plasmids were most likely acquired by conjugal transfer from *Haemophilus parainfluenzae* which carries a similar plasmid (Elwell et al 1977; Roberts et al 1977).

The β-lactamase-producing plasmids of *N. gonorrhoeae* are named Asia, Africa, Toronto, Rio, Nimes, New Zealand and Johannesburg types (Dillon et al 1999; Pagotto et al 2000) (Muller et al 2011). Only the Asia, Africa and the Toronto-type plasmids have been associated with epidemic outbreaks (Dillon and Yeung 1989). These plasmids are related and the structural diversity seen in gonococcal β-lactamase producing plasmids is due to the presence of several deleted or repeated sequences (Pagotto et al 2000). A novel β-lactamase-producing plasmid, a variant of prototype Asia plasmid was identified in gonococcal isolates from Johannesburg, South Africa (Fayemiwo et al 2010; Muller et al 2011).

Plasmid-mediated tetracycline resistance in *N. gonorrhoeae* isolates is due to the presence of the *tetM* gene. This plasmid is the result of the insertion of a streptococcal *tetM*-like sequence into the endogenous gonococcal 24.5-MDa conjugative plasmid to produce 25.2-MDa plasmid (Morse et al 1986; Knapp et al 1988). The TetM protein binds to the ribosome resulting in release of tetracycline from the ribosome and conferring a high level of resistance to tetracycline (Burdett 1986, 1991; Knapp et al 1988). *tetM* plasmids are differentiated into American (1600bp) or Dutch type (700bp) on the basis of the size of polymerase chain reaction (PCR) product (Ison et al 1993; Xia et al 1995; Greco et al 2003).

Chromosomally-mediated resistance (CMR) of *N. gonorrhoeae* is more complex and caused by mutations or modifications in various genes (Table 1.1). The accumulation of chromosomal mutations may confer resistance to multiple antibiotics or confer higher levels of

Table 1.1 Genes with selected mutations commonly implicated for antimicrobial resistance in *N. gonorrhoeae*

Genes	Known mutations influencing antibiotic susceptibility	Antibiotic resistance phenotype	References
penA	Non-mosaic; point mutations Mosiac *penA* ; homologous recombination between *N. gonorrhoeae penA* and *penA* of commensal *Neisseria*	Penicillin[a] and extended spectrum cephalosporins[b]	Allen et al 2011, 2013; Ohnishi et al 2011b; Lee et al 2010; Liao et al 2011; Zhao et al 2009; Whiley et al 2007a; Ito et al 2005; Olesky et al 2002; Ameyama et al 2002
mtrR	Adenine deletion (A-) or a dinucleotide insertion (TT+) in promoter A39T and G45D in DNA binding domain H105Y, E202G in dimerization domain Mutation combinations e.g. A-;G45D*	Erythromycin[c], azithromycin[d], penicillin, tetracycline[e], quinolones and extended spectrum cephalosporins	Allen et al 2011; Lee et al 2010; Liao et al 2011; Zhao et al 2009; Lindberg et al 2007; Warner et al 2008; Veal et al 2002; Hagmen et al 1995; Shafer et al 1995
porB	G120K; A121D* in loop 3	Penicillin, tetracycline and extended spectrum cephalosporins	Allen et al 2011; Lee et al 2010; Liao et al 2011; Zhao et al 2009; Lindberg et al 2007; Allen et al 2011; Olesky et al 2002
ponA	L421P	Penicillin	Ropp et al 2002
pilQ2	E666K	Penicillin	Zhao et al 2005
rspJ	V57M	Tetracycline	Allen et al 2011; Hu et al 2005
23S rRNA	C2611T and C2599T	Erythromycin and azithromycin	Ng et al 2002; Galarza et al 2010; Chisholm et al 2010; Katz et al 2012
gyrA	S91F, D95A	Quinolones[d]	Bellan et al 1994; Deguchi et al 1995; Tree et al 1998;Yang et al 2006; Starnino et al 2010
parC	D86N, S87N/ R	Quinolones	Bellan et al 1994; Deguchi et al 1995; Tree et al 1998;Yang et al 2006; Starnino et al 2010

*: A-;G45D- adenine deletion in *mtrR* promoter and G45D substitution in MtrR DNA binding domain are present together.
a: clinical level of penicillin resistance is due to additive effect of mutations in *penA, mtrR, porB, ponA* and *pilQ2*
b: reduced susceptibility or resistance to extended spectrum cephalosporins requires combined presence of *penA, mtrR* and *porB*
c & d: resistance to erythromycin and azithrmycin is due to mtrR and 23S rRNA
c: tetracycline resistance is due to combined presence of mutations in *penA, mtrR, porB* and *rspJ*
d:quinolone resistance requires mutations both in *gyrA* and *parC*

resistance to single antibiotic (Sparling et al 1975; Dillon and Pagotto et al 1999; Ropp et al 2002). Generally, these mutations alter structure and function of the cell envelope (Shafer and Folster 2006).

Penicillin resistance: Penicillin resistance in *N. gonorrhoeae* develops after sequential acquisition of resistance determinants through homologous recombination with commensal *Neisseri*a or DNA uptake from closely related species (Fig 1.5). Of the four penicillin binding proteins (PBPs) identified in *N. gonorrhoeae*, PBP1 (*ponA*) and PBP2 (*penA*) are essential for cell viability and are the targets of β-lactam antibiotics action (Barbour 1981). PBP1 and PBP2 both possess transpeptidase activity and catalyze the formation of peptide cross-links between adjacent glycan strands of peptidoglycan (Barbour 1981; Powell et al 2009). PBP1 also has an N-terminal transglycosylase domain that polymerizes glycan strands (Barbour 1981; Powell et al 2009). PBP2 is a primary target of penicillin and inhibited at 10-fold lower penicillin concentrations compared to PBP1 (Barbour 1981; Powell et al 2009). A codon insertion of aspartic acid at amino acid position 345 and four (F504L, A510V, A516G, H541N) to eight mutations (F504L, A510V, A516G, H541N, P552V, K555Q, I556V, I566V) clustered in the C terminus of the PBP2, were found to lower the rate of acylation of PBP2 by penicillin G 16-fold which, in turn, resulted in reduced susceptibility to penicillin (Whiley et al 2007a; Powell et al 2009; Ohnishi et al 2011b).

mtrR encodes the multiple transfer resistance repressor (MtrR) which represses the expression of the MtrC–MtrD-MtrE efflux pump. The MtrC-MtrD-MtrE efflux pump exports hydrophobic agents, such as macrolide antibiotics, Triton-X 100 and capric and cholic acids outside the cell. It is also implicated in high level penicillin resistance (Veal et al 2002). Mutations in the promoter and structural gene of *mtrR* result in overexpression of the efflux pump and increased efflux activity. The most common mutations associated with MtrR are: adenine deletion (A-) in the 13bp inverted repeat region between the -10 and -35 hexamers of the *mtrR* promoter, and A39T or G45D mutations in the structural gene of *mtrR* (Hagman et al 1995; Warner et al 2008). The mutation in the promoter abrogates *mtrR* transcription and RNA polymerase is not able to interact with the *mtrR* promoter (Lucas et al 1997). This overexpresses the efflux action of the MtrC–MtrD-MtrE pump.

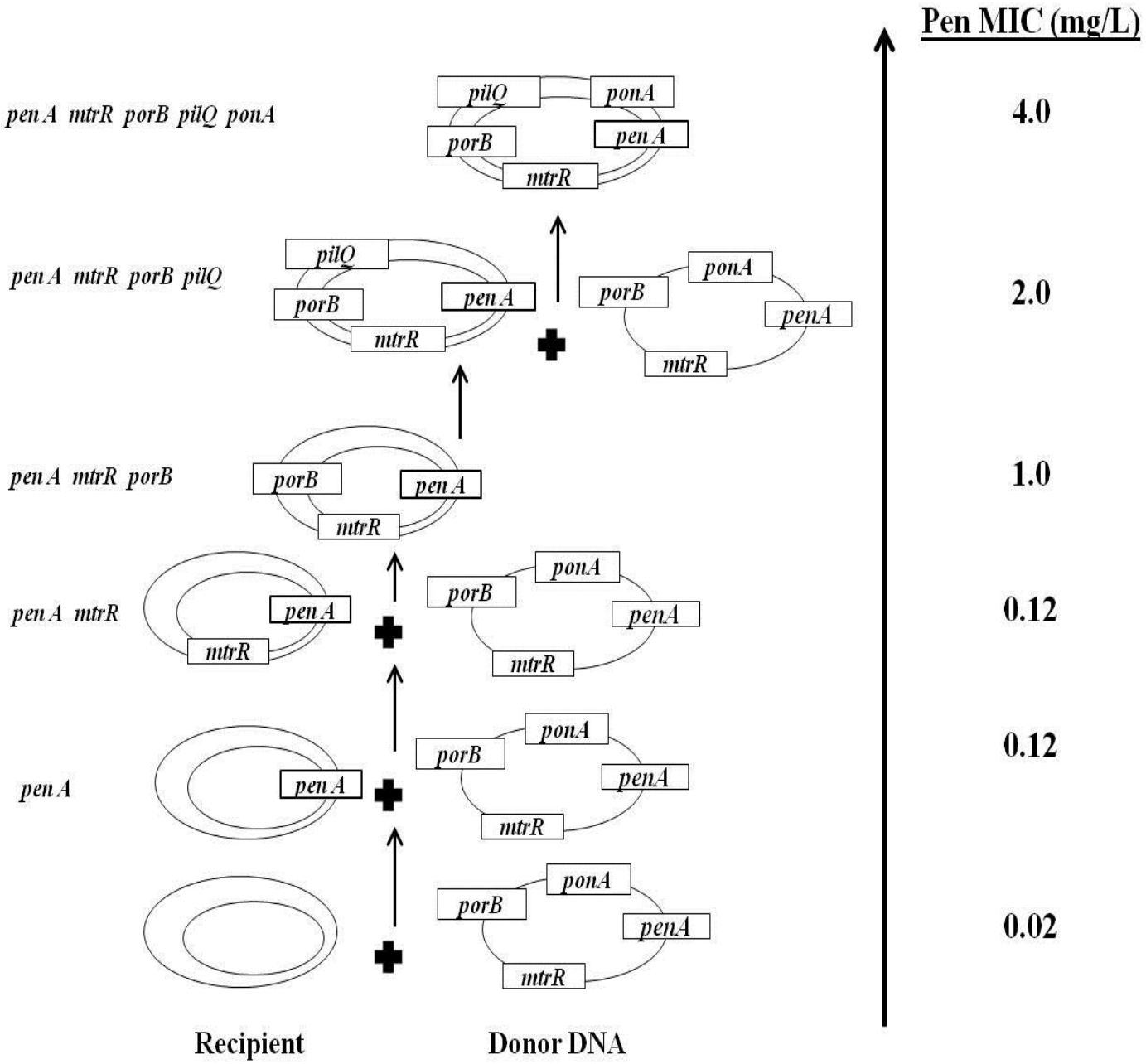

Fig. 1.5 Stepwise acquisition of penicillin resistance genes in *N. gonorrhoeae*

Source: Ropp et al 2002.

A helix–turn–helix (HTH) DNA binding motif exists between amino acid residues 32–53; missense mutations at residues 39 (A39T) or 45 (G45D) can enhance gonococcal resistance to antimicrobials such as macrolides, β-lactams, tetracyclines and quinolones. Presumably these substitutions abrogate MtrR binding to the target DNA upstream of *mtrR* (Hagman and Shafer, 1995; Shafer et al 1995). Other mutations that cause radical amino acid replacements in the centre of coding sequence (H105Y) or the C-terminal domain can also impact MtrR function, possibly by altering MtrR multimer formation (Warner et al 2008). Although, the roles *mtrR* mutations have been described in conferring AMR, the effects of combinations of mutations present together in the different regions of *mtrR* e.g. promoter (A-) or DNA binding motif (G45D) or multimeric region (H105Y) have not been experimentally proven to determine their contribution to gonococcal AMR.

The gene *porB* encodes a major outer membrane porin protein. Mutations in PorB cause a decrease in the influx of antibiotics to the periplasm (Fig 1.6) (Olesky et al 2002). PorB allows the passage of small molecules, such as β-lactams and tetracyclines, through the outer membrane (Danielsson et al 1986). PorB proteins are vital for cell viability, pathogenesis (Hook and Handsfield 1999) and antimicrobial susceptibility (Olesky et al 2002, 2006). Gonococcal PorB is a transmembrane protein comprising eight highly variable loops separated by nine conserved regions (van der Lay et al 1991; Carbonetti & Sparling 1987).

The surface exposed-loop regions of PorB have a high frequency of amino acid variations (Ison et al 1988; Smith et al 1995). *N. gonorrhoeae* has two *porB* alleles, *porB*1a (PIA) and *porB*1b (PIB) (Cooke et al 1998; Gill et al 1994). These alleles encode one of the two PorB proteins, either PorB1a or PorB1b, which are singly present in the bacterium (Fig 1.6) (Gotschlich et al 1987). Hybrid PI forms have occasionally been identified from clinical isolates by serological testing (Shinners & Catlin 1988). *porB*1a lacks sequence fragments in the loop 5 coding regions (van der Ley et al 1991; Derrick et al 1999).

Amino acid substitutions at Gly-120 and Ala-121 of loop 3 of PIB are associated with decreased susceptibility to β-lactams and tetracycline (Olesky et al 2002). The identical

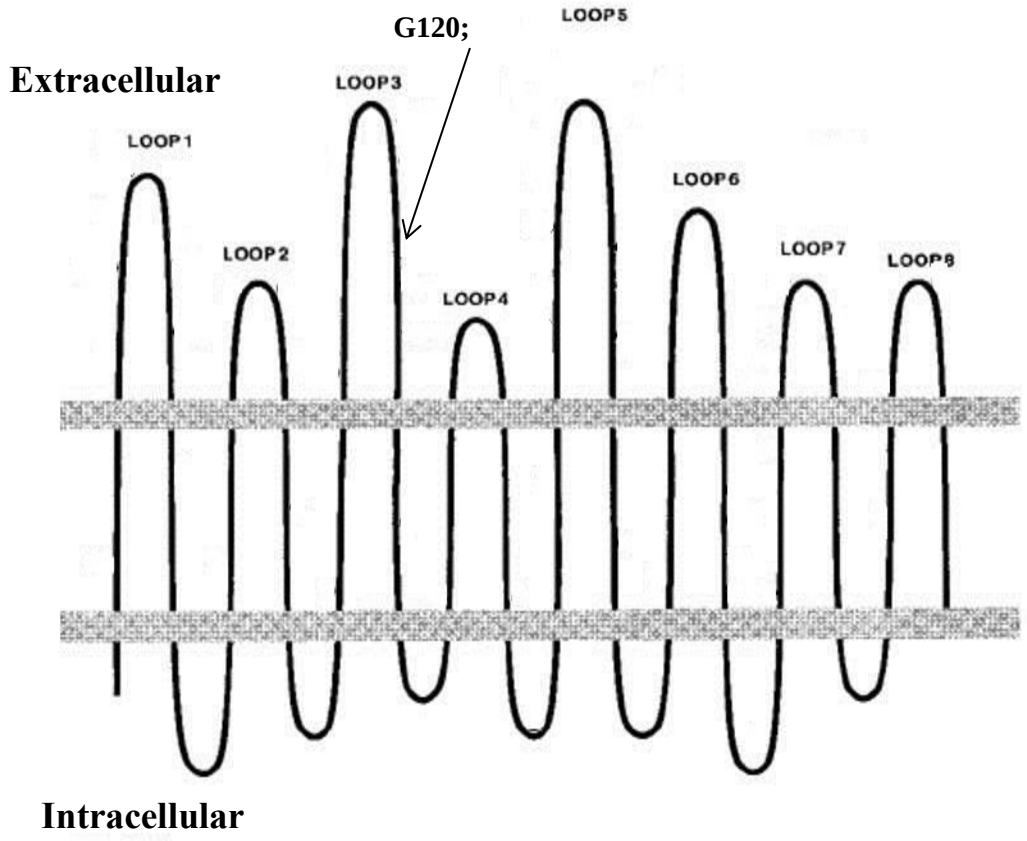

Fig. 1.6 Schematic presentation of *N. gonorrhoeae* PorB.

The length of PorB differs between PIB (~348 amino acids) and PIA (~328 amino acids), respectively. There are 8 surface exposing loops (1– 8) spanned by inter-space regions (van der Lay et al 1991). The amino acids G120 and A121 locate at loop 3. DNA sequences used in phylogenetic analysis include coding regions of loop 1 to 7. DNA sequences in *N. gonorrhoeae* multi-antigen sequence typing (NG-MAST) analysis included coding sequences for Loop III to Loop VII.

Source: Posada et al 2000.

mutations in PIA do not significantly change the susceptibility of *N. gonorrhoeae* to penicillin (Olesky et al 2002). The increase in resistance due to the *porB* allele occurs only in those strains, which already harbor *mtrR* mutations (Cooke et al 1998). A limited number of population studies of *N. gonorrhoeae* strains have determined the potential role of the specific mutations in *porB*, i.e. substitutions with charged amino acids at G120 and A121, in imparting penicillin resistance (Malakhova et al 2006; Allen et al 2011). Sun et al (2010) in a population study reported that deletions of A121 and N122 in PIB are associated with high levels (MIC=4-8 mg/L) of chromosomal penicillin resistance.

A single amino acid substitution in *ponA* (*ponA1* allele; L421P) produced a PBP1 with reduced affinity for penicillin (Ropp et al 2002). The presence of L412P alone does not affect the penicillin susceptibility of *N. gonorrhoeae* (Ropp et al 2002). *ponA1* contributes to high-level penicillin resistance in *N. gonorrhoeae* when present along with other penicillin resistance determinants such as *penA, mtrR* and *porB* (Ropp et al 2002). The significance of *ponA1* mutations in contributing to the observed clinical levels of penicillin resistance is not established through *N. gonorrhoeae* population studies and requires investigation (Allen et al 2011).

PilQ, a member of the secretin family of proteins, is a major component of the gonococcal outer membrane (Newhall et al 1980). PilQ is required for formation of the type IV pilus, which is involved in twitching motility, cell attachment and invasion of epithelia, and DNA transformation (Tonjum and Koomey 1997). PilQ multimers form a pore around the pilus in the outer membrane of *N. gonorrhoeae* allowing antibiotics to diffuse into the periplasm. A *pilQ2* missense mutation (E666K) alters pilQ multimeriazation, thereby blocking the entry of antibiotics by destabilizing the pore (Zhao et al 2005). The *pilQ2* mutation appears to increase cellular resistance to hydrophilic antibiotics (penicillin and tetracycline) but not to hydrophobic antibiotics (erythromycin and azithromycin) (Ropp et al 2002; Sparling et al 1975).

The presence of all of the resistance determinants (*penA*, *mtrR*, *penB*, *ponA1* and *pilQ2*) is essential for conffering a clinical level of penicillin resistance (MIC≥2mg/L) in *N. gonorrhoeae* isolates (Ropp et al 2002). The recipient susceptible strains of *N. gonorrhoeae* i.e FA19 transformed with all the known resistance determinants present in *N. gonorrhoeae* strains

with high-level penicillin resistance do not have the same penicillin MICs as associated with donar resistant gonococci (Lindberg et al 2007). This suggests that the individual resistance determinants are incapable of effecting high level resistance and therefore complex interactions between them are required to exert resistance effects (Shafer et al 2010). In essence, penicillin resistance mechanisms in *N. gonorrhoeae* require further understanding.

Reduced susceptibility to extended spectrum cephalosporins: The known molecular determinants of reduced susceptibility to extended spectrum cephalosporins are the same as those conferring penicillin resistance (Table 1.1). But, the exact mechanisms in *N. gonorrhoeae* of reduced susceptibility to extended spectrum cephalosporins remain undetermined. The mechanism(s) of reduced susceptibly of *N. gonorrhoeae* to extended spectrum cephalosporins are complex, since different combinations of mutations, within a gene or in different genes, have been observed in these isolates (Tanaka et al 2006; Lindberg et al 2007; Zhao et al 2009; Lee et al 2010; Liao et al 2011; Allen et al 2011; Ohnishi et al 2011b). Mutations in *penA* (PBP2) can provide reduced susceptibility to extended spectrum cephalosporins. The transpeptidase domain of PBP2 is a hyper-variable region (amino acid residues 340 to 581). On the basis of mutations present in this region, different mutation pattern numbers have been assigned to PBP2 (Ito et al 2005; Whiley et al 2007a; Lee et al 2010; Liao et al 2011; Allen et al 2011; Ohnishi et al 2011b). Thirty-nine polymorphism patterns (I-XXIX) of PBP2 between AA340-AA575 have been described (Ito et al 2005; Lee et al 2010; Whiley et al 2007a, b; Ohnishi et al 2011b; Martin et al 2012).

The decreased susceptibility to extended spectrum cephalosporins in gonococci is due to alterations of *penA* (Fig 1.7) The acquisition of a *penA* mosaic alleles, which are thought to be acquired by gonococci from commensal *Neisseria* strains by *in vivo* transformation and homologous recombination, is considered as the most common mechanism of decreased susceptibility to extended spectrum cephalosporins (Spratt 1988; Ameyama et al 2002; Ito et al 2005 Allen et al 2011; Tomberg et al 2010; Ohnishi et al 2011b; Unemo et al, 2010, 2011, 2012a,b). The amino acid alterations A501V/T in PBP2 increase MICs to cephalosporins (Tomberg et al 2010; Ohnishi et al 2011b; Unemo et al 2010, 2011, 2012a,b).

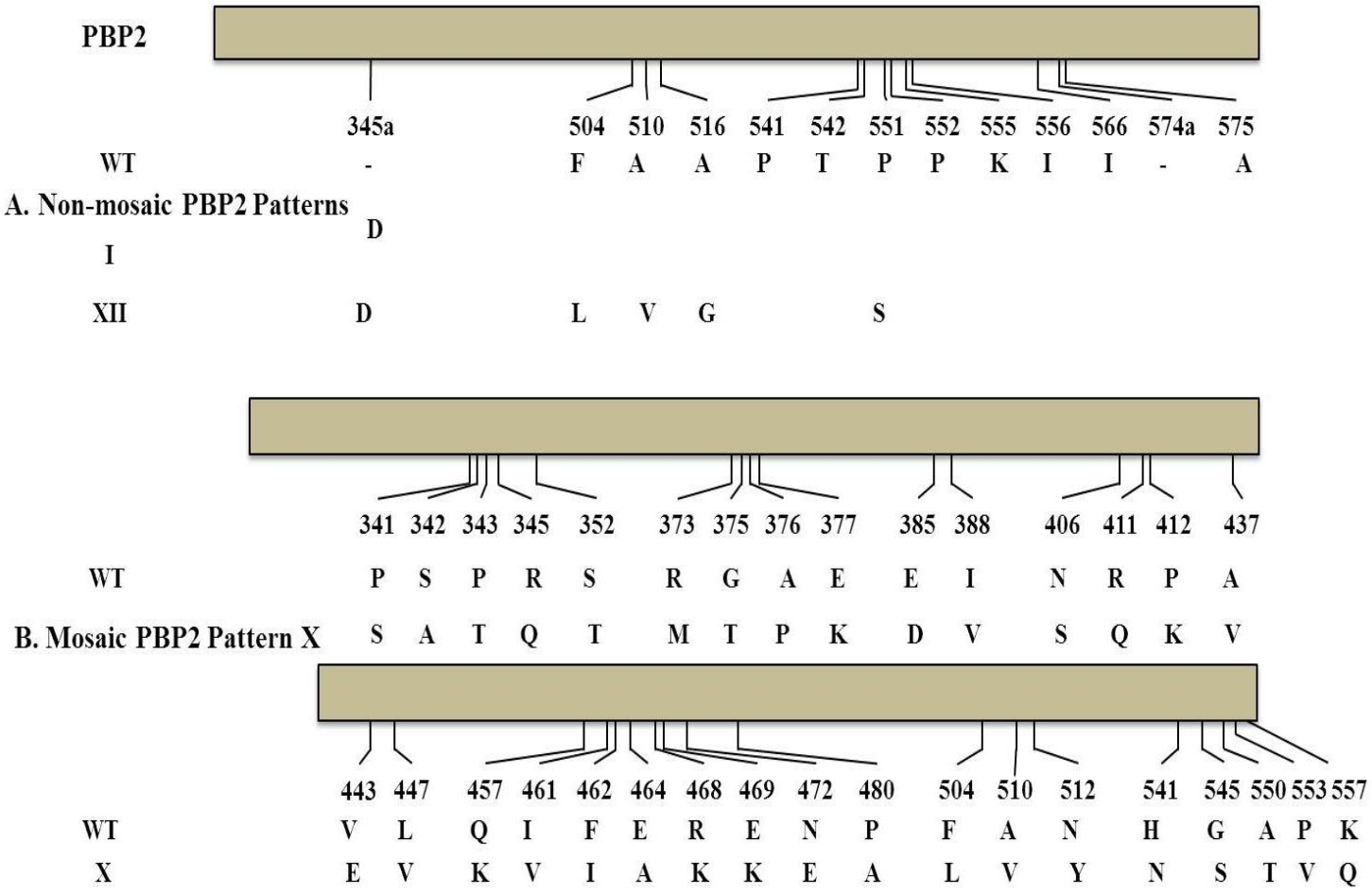

Fig. 1.7 Schematic presentation of non-mosaic and mosaic patterns of Penicillin Binding Protein (PBP2) in *N. gonorrhoeae*. Arrangements of point mutations on C-terminal transpeptidase domain of PBP2 were shown (Dougherty et al 1980). A) Non-mosaic PBP2 pattern I & IX and B) mosaic PBP2 pattern X. (Ito et al 2004; Whiley et al 2007; Ohinishi et al 2011b).

Adapted and modified: Powell et al 2009.

The first report of a *N. gonorrhoeae* strain with reduced susceptibility to cefixime demonstrated that the bacterium possessed a *penA* allele with 50-60 amino acid changes in PBP2. These were later described as mosaic PBP2 pattern X (Ameyama et al 2002; Ito et al 2005). *N. gonorrhoeae* strain H041 isolated in Japan, which is associated with high level of resistance to cefixime and ceftriaxone as well as to multiple antibiotics and treatment failure with ceftriaxone, was shown to carry mosaic allele $penA_{H041}$. This new PBP2 pattern has 8 new amino acid substitutions not observed in other mosaic *penA* alleles (Ohnishi et al 2011b; Goire et al 2011).

The oro-pharynx is thought to be a key site for the development of novel mechanisms of gonococcal resistance as a result of genetic exchange between commensal *Neisseria* spp. and *N. gonorrhoeae* (Barry & Klausner 2009). The mosaic alleles of *penA*, are generated by recombination of regions of *N. gonorrhoeae penA* genes with the *penA*s of other *Neisseria* spp.colonizing the oro-pharynx, such as *N. sicca*, *N. perflava, N. cinerea*, and/or *N. flavescens* (Ameyama et al 2002; Ito et al 2005; Whiley et al 2007a).

Various population studies showed that both *penA* non-mosaic patterns and the mosaic patterns are associated with reduced susceptibility to extended spectrum cephalosporins (Ito et al 2005; Whiley et al 2007a, 2010a; Lee et al 2010; Liao et al 2011; Allen et al 2011; Ohnishi et al 2011b). The importance of non-mosaic patterns, or mosaic patterns, other than pattern X and $penA_{H041}$ was not verified (Zhao et al 2009; Ohnishi et al 2011b).

Evidence for the role of *ponA* in susceptibility to extended spectrum cephalosporins is conflicting. Transformation studies of *N. gonorrhoeae* showed that *ponA* (PBP1) did not contribute to reduced susceptibility to extended spectrum cephalosporins (Zhao et al 2009). Liao and others (2011) did not observe any significant association of *ponA* with extended spectrum cephalosporin reduced susceptibility. In contrast, other population study reports for gonococcal AMR showed that *ponA* mutations were present in *N. gonorrhoeae* isolates with reduced susceptibility to cephalosporins (Lindberg et al 1997, Lee et al 2010; Allen et al 2011). Therefore, the role of *ponA* in contributing to reduced susceptibility to extended spectrum cephalosporins remains to be determined.

Population studies of AMR in *N. gonorrhoeae* isolates have shown that *mtrR* mutations A-, A-;G45D, and A39T;H105Y and G45D;H105Y are associated with reduced susceptibility to extended spectrum cephalosporins (Lindberg et al 2007; Lee et al 2010; Allen et al 2011; Liao et al 2011). Transformation studies have indicated that the *mtrR* mutation combinations A-;G45D contributed to reduced susceptibility to cefixime and ceftriaxone (Zhao et al 2009). Transformation studies are required to determine the role of other *mtrR* mutation combinations such as A39T;H105Y, G45D;H105, A-;H105 in conferring reduced susceptibility to extended spectrum cephalosporins.

PorB mutations (G120K;A121D and G120K;A121N) known to confer penicillin resistance also contribute to reduced susceptibility to extended spectrum cephalosporins in *N. gonorrhoeae* strains (Lindberg et al 2007; Lee et al 2010; Allen et al 2011). Also G120K;A121D mutations together with MtrR mutations (A-;G45D) have been experimentally demonstrated to contribute to reduced susceptibility to extended spectrum cephalosporins (Zhao et al 2009). However, in a recent study, Liao et al (2011) reported no significant association between specific PorB mutations and reduced susceptibility to extended spectrum cephalosporins. Alterations in *pilQ*2 have not been shown to contribute to the reduced susceptibility of *N. gonorrhoeae* isolates to extended spectrum cephalosporins (Whiley et al 2010b).

The mechanisms of reduced susceptibility to extended spectrum cephalosporins appear to differ for the oral extended spectrum cephalosporin, cefixime and parenteral extended spectrum cephalosporin, ceftriaxone. It has been suggested that cefixime does not diffuse into the periplasm through porin channels of *N. gonorrhoeae*. This can be attributed to the double negative net charge on cefixime compared to a single negative in ceftriaxone (Zhao et al 2009). The increase in net negative charge may affect the permeation of cefixime into *N. gonorrhoeae* (Zhao et al 2009). The majority of cefixime resistance is conferred by the *penA* allele, with only small contributions attributable to *mtrR* and *porB*, whereas ceftriaxone resistance is nearly equally dependent upon *mtrR* and *porB* (Zhao et al 2009).

Quinolone resistance: The quinolones (e.g. ciprofloxacin; ofloxacin) block DNA replication and repair in bacteria by targeting DNA gyrase (*gyrA*) and topoisomerase IV (*parC*) (Walsh, 2003). Quinolone resistance in *N. gonorrhoeae* is caused by point mutations arising in *gyrA* and *parC* within their quinolone resistance-determining regions (Table 1.1) (QRDRs, Belland et al 1994; Deguchi et al 1995, 1996; Trees et al 1998; Yang et al 2006; Starnino et al 2010). Drug permeability and drug efflux mechanisms also confer cross-resistance to structurally unrelated antibiotics (Nikaido 1994). In *N. gonorrhoeae* mutations affecting drug permeation and drug efflux, such as *mtrR* mutations, contribute to fluoroquinolone resistance (Starnino et al 2010).

Mutations in *gyrA* alone provide low (MIC=0.25mg/L) to intermediate (MIC=0.5mg/L) levels of resistance to ciprofloxacin (Belland et al 1994). A high level of quinolone resistance (MIC=4.0-16.0mg/L) also requires *parC* mutations (Belland et al 1994). A number of GyrA and ParC amino acid alteration patterns have been identified (Trees et al 1999; Yang et al 2006; Starnino et al 2010; Uehara et al 2011). The GyrA Ser91Phe/Asp95Ala and ParC Asp86Asn patterns are most commonly reported around the world (Trees et al 1999; Yang et al 2006; Starnino et al 2010).

Mutations in *mtrR,* along with QRDR mutations, have been observed in ciprofloxacin resistant (MIC= 1->32mg/L) *N. gonorrhoeae* isolates (Starnino et al 2010; Chen et al 2010). However, no genetic study has been conducted to determine the role of *mtrR* mutations in quinolone resistance. Quinolone resistance in *N. gonorrhoeae* has been limited primarily to QRDR mutations but the contribution of efflux pumps such as MtrC-MtrD-MtrE in decreasing *N. gonorrhoeae* susceptibility to quinolones is not fully established.

Azithromycin resistance: Azithromycin is effective against *N. gonorrhoeae* but the recommended dose (2g) is not well tolerated and causes gastrointestinal distress (Tapsall et al 1998a; CDC 2002, 2010). Clinical strains displaying intermediate susceptibility or resistance to azithromycin emerged first in the 1990's and new variants continue to be found (Dillon et al 2001a; Dillon et al 2001b; Sosa et al 2003; Dillon et al 2006; Palmer et al 2008; Galarza et al 2009; Starnino et al 2009; Chisholm et al 2009, 2010). Azithromycin resistance in *N. gonorrhoeae* can arise by a number of mechanisms: mutations either in the promoter region

(adenine deletion, A- or a dinucleotide insert, TT+) of *mtrR* or in the *mtrR* structural gene (A39T, G45D and H105Y), are known to cause low-level macrolide resistance in gonococcal strains (Table 1.1) (Zarantonelli et al 1999; Cousin et al 2003; Warner et al 2008). The presence of *mef* gene encoding a membrane-bound efflux protein, MacA-MacB efflux pump system, in *N. gonorrhoeae* can confer azithromycin resistance (Luna et al 2000). Alterations in 23S rRNA or the presence of methylase enzymes (encoded by *ermA*/*ermB*/*ermC/ermF*) prevents azithromycin binding to 23S rRNA, the target for azithromycin (Chung et al 1999; Roberts et al 1999; Ng et al 2002; Galarza et al 2010; Chisholm et al 2010). Recent reports suggest that emerging low (MIC=8 mg/L) to high (MIC≥ 256mg/L) azithromycin resistance in *N. gonorrhoeae* is due to mutated 23SrRNA alleles (Table 1.1) or the presence of rRNA methylases (Galarza et al 2010; Chisholm et al 2010; Katz et al 2012). These reports are important, particularly since azithromycin is one of the antibiotics in the recommended combination therapy for the treatment of gonorrhea (CDC 2012; HPA 2011; PHAC 2011c).

Spectinomycin resistance: Spectinomycin is used to treat infections due to β-lactamase producing *N. gonorrhoeae* or in treating the patients who cannot tolerate cephalosporins and quinolones (Boslego et al 1987; Kubanova et al 2010; CDC 2010). Frequent use of spectinomycin may lead to the emergence of resistance. Therefore monitoring of its use and of the treated patients is crucial (Boslego et al1987; Kubanova et al 2010). Spectinomycin acts on the 30S ribosomal subunit, inhibiting translocation of peptidyl tRNA (WHO 2003). Spectinomycin resistance can develop due to mutations G1064C and C1192U in helix 34 of 16S rRNA (Galimand et al 2000; Dillon 2007). Recently, a deletion of codon 27 and a K28E alteration in the ribosomal protein 5S has also been shown in *N. gonorrhoeae* isolated with high level of spectinomycin resistant (MIC>1024 mg/L) (Unemo 2012b). The role of other resistance mechanisms or mutations such as *mtrR* mutations, which contribute to clinical levels of resistance to different antibiotics in *N. gonorrhoeae*, may also be worth investigating in spectinomycin resistance.

Tetracycline resistance: Some of the resistance determinants implicated for chromosomal penicillin resistance also impart resistance to tetracycline. Intermediate levels of tetracycline resistance (MIC≥1 mg/L) may develop due to mutations in the promoter or structural gene of

mtrR. Substitution of charged amino acids (i.e. arginine, lysine, aspartic acid and histidine) at positions G120 and A121 position in PorB may also cause tetracycline resistance (Table 1.1) (Olesky et al 2002; Pan and Spratt 1994). High-level chromosomal resistance to tetracycline (MIC=6 mg/L) may be caused by a mutation in *rpsJ1* together with *mtrR* and *porB* mutations (Hu et al 2005). A mutation (V57M) in the *rpsJ* gene encoding ribosomal protein S10 modulates the rRNA-binding site for tetracycline, thus lowering the affinity of the antibiotic for the ribosome (Table 1.1) (Hu et al 2005; Allen et al 2011). Deletions at positions A121 and N122 in PIB were shown to be associated with MICs of 4-16 mg/L of chromosomal tetracycline resistance (Sun et al 2010).

1.2.3 Impact of Antimicrobial Resistance on Treatment Guidelines for Gonococcal Infections

Sulfanilamides, introduced in 1936, were the first effective anti-gonococcal antibiotics (Lees 1946). Subsequent studies showed that sulphanilamides cure 80 to 90% of gonorrhea cases (Kampmeier 1983). However, by the end of the 1950, over 90% of *N. gonorrhoeae* isolates were resistant to sulphonamides (Kampmeier 1983; Lewis 2010). A combination of trimethoprim and sulfamethoxazole was used to treat gonorrhea till the 1970's (Austin et al 1973; Lawrence et al 1973) until resistance developed to these agents.

Penicillin was first used to treat gonococcal urethritis in 1943 (Van Slyke et al 1943). Penicillin remained the antibiotic of choice for the treatment of gonorrhea for the next 40 years (Van Slyke et al 1943; Lewis 2010). The emergence of high-level, plasmid-mediated resistance to penicillin in 1976 marked the end of the use of penicillin for the treatment of gonorrhea (Percival et al 1976; CDC 1976). Eventually, treatment with penicillin was discontinued in 1985 in most regions (Dillon & Pagotto 1999).

Tetracyclines were used initially to treat gonorrhea in patients who were allergic to penicillin. Tetracycline is still in use today for treating suspected, or proven, chlamydial co-infection (PHAC 2008b, 2010b; CDC 2010). However, gonococci became less susceptible to tetracycline shortly after it was introduced to gonorrhrea treatment guidelines. This occued due

to rapid emergence and spread of gonococcal strains with plasmid mediated tetracycline resistance (Reyn et al 1958). This antibiotic was no longer recommended for the treatment of gonorrhea when plasmid-mediated tetracycline resistant isolates were identified (Roberts et al 1988; CDC 1985; Dillon & Pagotto 1999; Greco et al 2003). Plasmid-mediated tetracycline resistant *N. gonorrhoeae* (TRNG) were first reported in the USA in 1985 and then in the Netherlands (CDC 1985; Roberts et al 1988). TRNG are now widespread, probably as a result of widespread use of tetracyclines in STI management (Lewis 2010).

Erythromycin has limited activity against *N. gonorrhoeae* and is not recommended for the treatment of gonorrhea (Brown et al 1977) except if penicillin is not tolerated and in pregnant women (Dillon et al 1998). Azithromycin is more active against gonococci and has the advantage of single-dose oral treatment. Azithromycin has been used in some Latin American countries for the primary treatment of *N. gonorrhoeae* infections (Starnino et al 2012), however in North America, azithromycin is not recommended as a first-line treatment option for gonorrhea owing to concerns over development of resistance and side effects of the recommended 2 g dose (Young et al 1997; WHO 2003). The earliest reports of decreased susceptibility and resistance to azithromycin came from Latin America in the mid to late 1990s (Dillon et al 2006; Sosa et al 2003; Dillon et al 2001a). Azithromycin resistant gonococci were isolated from Canada and the USA (Ng et al 2002; Johnson et al 2003). High-level azithromycin resistance (MIC>256 mg/L) to *N. gonorrhoeae* has been reported from Scotland (Palmer et al 2008), Argentina (Galarza et al 2009; 2010), the UK (Chisholm et al, 2009 & 2010) and Italy (Starnino et al 2009).

Fluoroquinolones, such as ciprofloxacin and ofloxacin, were widely used to treat gonorrhoea from the mid-1980s onwards (Lewis 2010). In 1993, oral fluoroquinolones were recommended for gonorrhea treatment in the USA (CDC 1993). Trends in treatment recommendations for gonorrhea in Canada have been similar to those in the USA (PHAC 2006). Initially, low doses of 250 mg were prescribed but, by 1990, treatment failures were reported and recommended treatment dose was raised to 500 mg (Gransden et al 1990; Ison et al 1998; Ray et al 2005). However, the emergence of quinolone-resistant *N. gonorrhoeae* (QRNG) became a concern in 1991 in the US and in the Asia-Western Pacific regions (Iverson et al 2004; Tapsall et

al 1998b; Tapsall 2002, 2005, 2006). Since then, the rates of QRNG have continuously increased and have been reported internationally (Yang et al 2006; Cole 2010, 2011; CDC 2009, 2011; Starnino et al 2010; Uehara et al 2011; Tanaka et al 2011; WHO-WPR 2012). Quinolones were not recommended as first-line gonococcal treatment in most Asia-Pacific countries in the mid-to-late1990s (Dan 2004; Lewis & Marumo 2009). In 2006, the US CDC abandoned quinolones as a drug of choice for treatment of gonorrhea (CDC 2007). Due to the rapid increase in rates of QRNG, quinolones such as ciprofloxacin and ofloxacin were shifted from the class of recommended drugs to the alternative options for the treatment of gonococcal infections in Canada (PHAC 2008a, b, 2011c).

Spectinomycin is an aminocyclitol antibiotic, closely related to the aminoglycosides and is a bacteriostatic agent. It was developed and marketed specifically for the treatment of gonorrhoea in the early 1960s (Lewis 2010). Spectinomycin resistance was first reported in a penicillin susceptible *N. gonorrhoeae* isolate in the Netherlands in 1967, and subsequently in a PPNG isolate acquired in the Philippines in 1981(Stolz et al 1975; Ashford et al 1981). However, shortly after its introduction and wide use in the mid-1980s, clinical treatment failures caused by spectinomycin resistant strains began to be appear in US military personnel in the Republic of Korea (Boslego et al 1987). Gonococcal resistance to spectinomycin has also been reported from the Western Pacific regions (WHO-WPR 2001) and from the Latin American countries (Dillon et al 2006).

Cephalosporins were first discovered in 1945 and modern variants are chemical modifications of this prototypic molecule (Barry & Klausner 2009). Because of global spread of QRNG and resistance to penicillin and tetracycline, extended-spectrum cephalosporins were recommended effective treatment of gonorrhea (Lewis 2010). Many countries recommend orally administered cefixime or injectable ceftriaxone as the antibiotics of choice, including Canada (2008a, b), the United States (CDC 2006, 2007, 2010), the United Kingdom (HPA 2005), China (Wang & Zhang 2007) and Japan (Jpn Soc STD 2006).

The recent reports of the emergence of strains with resistance or reduced susceptibility to extended spectrum cephalosporins, as well as treatment failures with this class of antibiotics,

have raised concerns about the use of extended spectrum cephalosporins (cefixime and ceftriaxone) as the first line of treatment for uncomplicated gonococal infections (Ito et al 2004, Tapsall et al 2009b; Ohinishi et al 2011; Unemo et al 2011b, 2012a,b; Martin et al 2012; Bolan et al 2012; Camara et al 2012; WHO 2012; Allen et al 2013). The spread of resistant *N. gonorrhoeae* strains, such as those isolated from Japan with high-level resistance to both cefixime and ceftriaxone as well as multiple antibiotics, may render *N. gonorrhoeae* untreatable in the near future (Ohinishi et al 2011; Dillon 2011). Ceftriaxone is essentially the last remaining treatment option and, worryingly, there have been cases of confirmed failure treating pharyngeal gonorrhea with ceftriaxone in Australia, Sweden and France (Tapsall et al 2009b; Unemo et al 2011c, 2012a, 2012c).

All these findings have led to a change in gonorrhea treatment recommendations in various countries. In Japan, cefixime has been replaced by ceftriaxone as a 1g dose given intravenously (Ohnishi et al 2011a). The CDC, USA, the Health Protection Agency (HPA), UK and Public Health Agency of Canada (PHAC) are now recommending ceftriaxone with at an increased dose of 250-500mg and 1g azithromycin as dual therapy for treatment of gonorrhea (CDC 2012; HPA 2011; PHAC 2011c).

Historically, thiamphenicol has been suggested in some countries as a viable alternate therapy regimen to penicillin in the treatment of infections with *N. gonorrhoeae*. It has not commonly been used in the treatment of *N. gonorrhoeae* since because cross-resistance to thiamphenicol was common in penicillin resistant isolates. Further, thiamphenicol can produce erythroid suppression (Dillon et al 1978a,b).

Aminoglycosides, kanamycin and gentamicin are rarely used for the treatment of gonorrhea because of their low activity against *N. gonorrhoeae* and their toxicity (Dillon 1992; WHO 2003). Kanamycin has been used to treat gonorrhea in Indonesia (Ieven et al 2003; Donegan et al 2006). Gentamicin is the national first-line treatment for gonorrhoea in Malawi and has also been used to treat gonococcal infections in Mongolia (Daly et al 1997; Lkhamsuren et al 2001; Brown et al 2010). Studies in Malawi have confirmed that gonococci remain susceptible to gentamicin after over 14 years of first-line use (Brown et al 2010). *In vitro* studies

had demonstrated that the European gonococcal population is also susceptible to gentamicin (Chisholm et al 2011). Resistance to other antibiotics does not confer resistance to gentamicin since its mechanism of action differs from other antibiotics recommended for the treatment of gonorrhea (Vakulenko & Mobashery 2003; Dowell & Kirkcaldy 2012). Gentamicin is widely available and inexpensive but requires parenteral administration. The risks of ototoxicity and nephrotoxity with a single-dose therapy (240-280 mg) appear to be minimal and are not observed in gonorrhea patients treated with a single dose of gentamicin (Barclay & Begg 1994; Dowell & Kirkcaldy 2012). Although the efficacy of gentamicin does not meet recommended treatment efficacy criteria (≥ 95% treatment efficacy), it could potentially prove to be a valuable treatment option for gonorrhea patients showing cephalosporin resistance or severe cephalosporin allergy (Dowell and Kirkcaldy 2012).

The new parenterally administered antimicrobial agents, ertapenem, a carbapenem and tigecycline, a glycylcycline (derived from tetracycline) are active against gonococci but require further testing against multidrug-resistant strains (Unemo et al 2012d; Livermore et al 2004; Deshpande et al 2001). Furthermore, a new fluoroketolide, solithromycin (CEM-101), has also been shown to have high in vitro activity against gonococci (Putnam et al 2010; Golparian et al 2012).

Gonococcal resistance to penicillin and tetracycline has been prevalent for several decades in Canada resulting in their elimination as treatment options (PHAC 2010b). Due to the rapid increase in QRNG, quinolones such as ciprofloxacin are no longer preferred drugs for the treatment of gonococcal infections in Canada. All these findings led to a change in Canadian treatment guidelines from ciprofloxacin to cefixime as the recommended drug for the treatment for gonorrhea, with ceftriaxone or spectinomycin as the alternative treatment (PHAC, 2008). In view of emergence of strains with resistance or reduced susceptibility to extended spectrum cephalosporins and treatment failures in other parts of the world, the Canadian treatment guidelines for gonorrhea were revised in 2011 (PHAC 2011c). In new treatment guidelines, Public Health Agency of Canada (PHAC 2011c), recommends the use of ceftriaxone (250 mg intramuscularly) or cefixime (800 mg orally) to treat gonococcal infections. Ceftriaxone is now being recommended as the preferred treatment for gonococcal infections in MSM. New

treatment guidelines also recommend use of single oral dose (1 gm) of azithromycin along with the recommended doses of extended spectrum cephalosprins to be used as empirical treatment to treat *Chlamydia* and gonococcl co-infections. It is important to note that PHAC still recommends that quinolones (ciprofloxacin 500 mg or ofloaxcin 400 mg as single dose orally) can be used as alternative treatment in those cases where the quinolone susceptibility of gonococcal isolates is demonstrated through antimicrobial susceptibility testing, or the QRNG prevalence is under 5%. Treatment guidelines issued by PHAC are followed all over Canada. Therefore, as per new recommendations ceftriaxone and azithromycin will be the drugs of choice to treat gonorrhea in SK and cefixime and ciprofloxacin can be used as alternatives drugs.

1.3 Understanding Transmission of *Neisseria gonorrhoeae* through Molecular Epidemiology

1.3.1 Overview of Typing Methods for *N. gonorrhoeae*

One of the important components of gonorrhea control programs is identification of sexual networks and strain clusters to understand transmission of *N. gonorrhoeae* in a population. A number of phenotypic and genotypic typing methods have been applied to determine the molecular epidemiology of gonorrhea (Sarafian and Knapp 1989; Unemo & Dillon 2011). The phenotypic characterizations of *N. gonorrhoeae* (Unemo & Dillon 2011) have included auxotyping (A) (Carifo & Catlin 1973; Ng & Dillon 1993), serovar determination (S) (Knapp et al 1984; Sandström et al 1982, 1984; Dillon et al 1987a, b), and the combination of A/S (Ng & Dillon 1993), as well as antibiogram determination, or a combination of these methods (Ng et al 1995). The use of phenotype based typing methods is limited due to the unavailability of reagents e.g. sera, the degree of technical expertise required e.g. auxotype, the relatively high cost and lack of discrimination (Unemo & Dillon 2011).

An ideal typing approach for epidemiological studies should have the characteristics of : 1) being able to type all strains in a species, 2) having enhanced discriminatory power while preserving the ability to demonstrate strain linkages, 3) reproducibility, 4) feasibility and applicability, 5) easy to perform and 6) cost (Olive & Bean, 1999; Unemo & Dillon 2011).

The discriminatory power of a typing method is its ability to distinguish unrelated strains. The "index of discrimination" (ID) indicates the probability of a typing method to differentiate two different isolates and has been used to characterize discriminatory abilities of different typing methods (Hunter & Gaston 1988). An ID of higher than 95% has been considered to be desirable in differentiating *N. gonorrhoeae* isolates with critical assessment of the confidence interval (Dillon et al 1993; Unemo & Dillon 2011). This index is influenced by sample size and the heterogeneity and clonality of the bacterial populations tested (Unemo & Dillon 2011).

Phenotypic Differentiation of *N. gonorrhoeae* Isolates

Antimicrobial susceptibility testing: This method involves the determination of MICs of *N. gonorrhoeae* isolates against one or many antibiotics. Strains are differentiated on their susceptibility or resistance profiles. Antimicrobial susceptibility testing is used for monitoring antibiotic susceptibility and for determining appropriate treatment recommendations (Dillon et al1978b, Dillon & Carballo, 1990; Dillon & Pagotto 1999). The results of the antimicrobial susceptibility testing can vary between different laboratories since uniformity does not exist in terms of susceptibility interpretation criteria, use of reference strains and lot numbers of antibiotics, and culture media used for testing. The antibiograms have low discriminatory power and are inappropriate for epidemiological studies of gonorrhea (Dillon et al 1993; Ng et al 1995; Unemo & Dillon, 2011). However, they are absolutely required for surveillance as this is the only way that AMR can be determined.

Auxotyping (A): Auxotypes of *N. gonorrhoeae* isolates are based on their different nutritional requirements for amino acids, purines, pyrimidines, and vitamins (Catlin 1973). Some nutritional components (e.g. cystine, cysteine) are required for all isolates of *N. gonorrhoeae* to grow, whereas other components are required by some isolates (Carifo & Catlin, 1973). Auxotyping differentiates *N. gonorrhoeae* isolates into various nutrient-requiring phenotypes, e.g. non-requiring (NR) or prototrophic, proline-requiring (P), arginine-hypoxanthine-uracil-requiring (AHU), and proline-citrulline-uracil-requiring (PCU) (Dillon et al 1986, 1987b). This method of typing also has low discriminatory ability, is time consuming and laborious, and requires higher

levels of training in both technical aspects and for interpretation of the test results (Dillon et al1987b, 1993, Unemo & Dillon 2011).

Serotyping (S) and Auxo/Sero (A/S) typing: Serotyping is based on the antigenic diversity of the major outer membrane porin proteins, known as the protein I (PI) molecules or PorB (Sandstrom et al 1982). Serovars are determined using coagglutination techniques (Sandstrom & Danielsson 1980) for detecting interactions between gonococcal antigens and panels of specific monoclonal antibodies (MAbs) (Knapp et al 1984). Two typing schemes were developed based on monoclonal antibodies (MAbs) generated against specific PI (Knapp et al 1984). One of these typing systems is the use of the Genetic system (GS) series of MAbs (Knapp et al 1984). Another set of PI-specific MAbs was designed as the Ph panel (Pharmacia; Bygdeman et al 1983; Kohl et al 1990). The utilization of the series of MAbs generated for this purpose, produces serotyping schemes with high discriminatory ability, especially when this is used in combination with auxotyping methods (Dillon et al 1987a, b, 1993; Unemo & Dillon 2011). However, use of this approach is limited by inconsistency in the interpretation of weakly positive results for some MAbs, the inaccessibility of some MAbs (GS), and the prevalence of non-serotypeable strains (Unemo et al 2003). Serovar determination has better discriminatory ability than auxotyping. It is fast, easy to perform, and relatively cost-efficient. The disadvantages include a reduction in the discriminatory ability compared to genotypic methods; this is largely due to the subjective interpretation of results, the low specificity of some MAbs, the increasing prevalence of non-serotypeable strains, and the emergence of new serovars over time due to the ongoing evolution of PorB (Unemo & Dillon 2011).

Multiple Locus Enzyme Electrophoresis: Multiple locus enzyme electrophoresis (MLEE) involves analysis of electrophoretic mobilities of housekeeping enzymes on starch gels (Selander et al 1986; Ng & Dillon 1993; De la Fuente & Vazquez 1992). MLEE indexes allelic variations based on banding patterns of multiple housekeeping enzymes. It may not be highly discriminatory (Ng & Dillon 1993; De la Fuente & Vazquez 1992). Differences in cultural growth conditions and enzyme productivity can make interpretation of results by MLEE difficult, especially if isolates tested are clones (Unemo & Dillon 2011).

DNA- Based Differentiation of *N. gonorrhoeae* Isolates

Genotypic methods, i.e. DNA sequencing methods, are currently the methods of choice for typing gonococcal isolates. Genetic methods are more discriminatory, reproducible, objective, and reliable compared to those based on phenotypes (Unemo & Dillon 2011). Selection for molecular typing systems is based on typeability (i.e. target sequence from each isolate can be amplified and analyzed), reproducibility, ease of interpretation, ease of use and greater discriminatory power (Maslow et al 1993). DNA-based typing methods include characterizing plasmids as well as determining polymorphisms in either a single locus or multiple loci over the entire genome, using a number of methods (Unemo & Dillon 2011).

DNA-based typing methods for characterizing *N. gonorrhoeae* strains can be broadly divided into two groups (Unemo & Dillon 2011): gel-based DNA-based typing methods and DNA sequence-based typing methods.

Gel based DNA typing methods

Gel based DNA typing methods include plasmid content analysis, restriction endonuclease (RE) analysis, restriction fragment length polymorphism (RFLP) determination using pulsed-field gel electrophoresis (PFGE), ribotyping, and Opa typing.

Plasmid Content Analysis: Analysis of plasmid content involves the characterization of either all plasmid content in the organism, or specifically targeted plasmids in *N. gonorrhoeae* isolates. Specific plasmids have been classified based on their phenotypes, molecular weights, or DNA sequences, including cryptic (no known phenotype) and conjugative plasmids, as well as resistance determinant-containing plasmids such as the *tetM*-carrying conjugative plasmid and the family of β-lactamase-producing plasmids (Unemo & Dillon 2011).

Most gonococcal strains carry a 2.6 megadalton (MDa) plasmid that is cryptic (i.e. no known function) and a 24.5 MDa transfer plasmid (Sox et al 1978). Plasmid analysis has been used to differentiate plasmid-mediated antimicrobial resistance including penicillinase-producing *N. gonorrhoeae* (PPNG) and plasmid-mediated tetracycline resistant *N. gonorrhoeae* (TRNG)

(Dillon & Yeung 1989; Ison et al 1993, Xia et al 1995; Greco et al 2003). β -lactamase plasmids can be typed by PCR (Dillon et al 1999). β -lactamase plasmids of *N. gonorrhoeae* can be detected in non-cultured specimens through real time PCR (Goire et al 2011). TRNG can be differentiated through PCR ((Ison et al 1993; Xia et al 1995; Greco et al 2003). The Uruguay TetM type exhibits a unique restriction pattern (Marquez et al 2002).

Typing methods based on analysis of plasmid content are easy to perform and are useful for investigating antimicrobial resistance and outbreak of plasmid-harbouring *N. gonorrhoeae* strains (Dillon et al 1993). However, plasmid content analysis has low discriminatory ability and may lack reproducibility, if plasmids are acquired or lost from isolates, hence it is not recommended as a routine typing method.

Restriction Endonuclease (RE) Analysis: Restriction endonuclease (RE) analysis involves cleavage of chromosomal DNA with type II (homodimers generating undivided and palindromic sites with 4–6 nucleotides in length) restriction endonucleases (REs; *SmaI, AvaII* or *HindIII*) and the resulting restriction fragments are analyzed by gel electrophoresis (Li & Dillon 1995). Type II REs cleave DNA into a unique set of fragments and provide a unique band pattern or restriction pattern or finger print of DNA examined (Southern 1979). RE analysis is highly specific in differentiating strains of bacteria but is both laborious and expensive (Li & Dillon 1995).

Restriction Fragment Length Polymorphism analysis and Pulsed Field Gel Electrophoresis: Restriction fragment length polymorphism (RFLP) analysis is based on digestion of the entire genome by low-frequency-cutting restriction endonucleases followed by separation of fragments by polyacrylamide gel electrophoresis (PAGE) (Falk et al 1984). This method is no longer commonly used because it is laborious and not suitable for high-throughput analysis since the results obtained require visual interpretation, leading to problems with reproducibility and inter-laboratory comparisons (Unemo & Dillon 2011).

The discrimination ability of RFLP analysis improves when combined with pulsed-field electrophoresis (PFGE) involving repeated change in the orientation of the electric field in gel

electrophoresis. It can resolve DNA fragments ranging in size up to 2000 kb (Li & Dillon 1995; Sor 1988). PFGE is based on digestion of the entire bacterial genome by rare-cutting restriction endonucleases (SpeI and/or BglII) has followed by separation of the resulting large (5 to 10 kb) DNA fragments in an agarose gel subjected to pulsed-field electrophoresis (Schwartz & Cantor 1984; Vollrath & Davis 1987; Li & Dillon 1995; Ng et al 1995; van Looveren et al 1999). PFGE is reproducible, increases discrimination between isolates in specific situations, and all *N. gonorrhoeae* isolates are typeable by this method (Unemo & Dillon 2011). However, PFGE lacks standard methods of comparison (Unemo & Dillon 2011).

Ribotyping: Ribotyping is based on the RFLP analysis of rRNA genes (Li & Dillon 1995; Ng & Dillon 1993). Ribotyping involves enzymatic digestion of the bacterial genome, electrophoresis, and visualization of the resulting banding patterns after hybridization with a ribosomal RNA probe (rRNA) (Pace et al 1986). Ribotyping has been evaluated for typing of *N. gonorrhoeae* strains (Li & Dillon 1995). However, this method has little applicability for *N. gonorrhoeae* strains, as it is laborious, has low discrimination, involves subjective interpretation of results, and therefore, not practical for use outside reference laboratories (Unemo & Dillon 2011).

Opa-typing: Opa-typing involves analysis of up to 11 *opa* genes coding for the neisserial colony opacity-associated (Opa) proteins (O'Rourke et al 1995; Palmer et al 2001; Ison 1998; Ward et al 2000). In Opa-typing, *opa* genes are PCR-amplified; PCR products are subjected to RE digestion with *TaqI* and *HhaI* and separation of fragments by gel electrophoresis; banding patterns are compared after gel electrophoresis (O'Rourke et al 1995). Opa-typing has high typeability, reproducibility and high discriminatory ability (Unemo & Dillon 2011). It has been used to define gonococcal populations, identification of strain clusters and in tracking strain transmission, and strain clustering in *N. gonorrhoeae* (Bilek et al 2007; Khaki et al 2009; Viscidi et al 2000; O'Rourke et al 1995; Unemo & Dillon 2011). Opa-typing is labor intensive and it is difficult to standardize and compare results obtained in different laboratories (Unemo & Dillon 2011).

Other typing methods: In amplified ribosomal DNA gene restriction analysis (ARDRA), a ribosomal gene fragment including part of the 16S rRNA gene, the 16S-23S rRNA spacer region and part of the 23S rRNA gene is PCR amplified, followed by digestion of PCR products with a

high-frequency-cutting restriction enzyme and subsequent gel electrophoresis analysis (Gu¨rtler and Stanisich 1996; van Looveren et al 1999).

Amplified fragment length polymorphism (AFLP) analysis is a PCR-based genome sampling technique that reproducibly generates fragment profiles of bacterial clones. Using each fragment size as an individual character, data can be reduced to binary form that can be stored in a database (Palmer & Arnold 2001; Spaargaren et al 2001).

Arbitrarily primed PCR (AP-PCR) or randomly amplified polymorphic DNA (RAPD) relies on annealing a single random primer at anonymous sites in the bacterial genome where it may be a perfect or an approximate match. AP-PCR results give an indication of genetic relatedness between isolates at multiple unknown locations in the bacterial chromosome (Hobbs et al 1999; Lawung et al 2010).

Multilocus variable-number tandem repeat (VNTR) analysis (MLVA) is gel based method and utilizes the variation in the number of tandem repeat sequences in different loci dispersed over the total genome (Schouls et al 2006). This variation is expressed in strain-specific numerical DNA profiles. MLVA results have good congruence with DNA sequence based typing methods (Heymans et al 2011, 2012). The advantage of MLVA analysis is that genetic data are translated into numerical profiles without the loss of valuable information (Kushnir et al 2012). The ID for MLVA is high and is comparable to DNA sequence based typing methods (Heymans et al, 2012; Kushnir et al 2012).

DNA sequence-based typing methods

DNA sequence analysis of various gene(s) is currently the method of choice for distinguishing *N. gonorrhoeae* isolates as it provides unambiguous and reproducible information, has high discriminatory power, and enables data to be stored or shared electronically, permitting reliable comparisons to be made between data obtained in different laboratories (Maiden 2006; O'Rouke et al 1995; Viscidi et al 2000).

Currently DNA sequence based molecular typing schemes are based on PCR amplification of either a single gene or multiple genes (Stefani and Agodi 2000; Dillon and Unemo 2011). These methods have greater discriminatory power, reproducibility, rapidity and high throughput facilitating inter-laboratory comparisons of results nationally and internationally. DNA sequence based typing methods coupled with sophisticated analysis of raw sequences such as phylogenetic analysis provide more comprehensive information of genetic relatedness of different STs (Unemo & Dillon 2011). DNA sequence based typing methods have been used to trace the transmission of individual *N. gonorrhoeae* strains within a community, to identify suspected clusters of cases of gonorrhea; to confirm epidemiological connections between patients (Hobbs et al 1999; Unemo et al 2002; Viscidi & Demma 2003; Choudhury et al 2006; Martin et al 2004, 2005; Lundba¨ck et al 2006; Liao et al 2008, 2009; Golparian et al 2010; Ilina et al 2010; Unemo & Dillon et al 2011), to investigate treatment failure (Ota et al 2009; Tapsall et al 2009b; Unemo et al 2010), to study population genetics (Fudyk et al 1999; Pe´rez-Losada et al 2007; Tazi et al 2010), tracing sexual contacts (Martin et al 2004; Unemo et al 2007; Bilek et al 2007; Abu-Rajab et al 2009), in medico-legal cases (Martin et al 2007), and, predicting antimicrobial resistance (Palmer et al 2008). DNA sequence-based typing methods routinely used for *N. gonorrhoeae* include full- or extended-length *porB* sequence analysis, *N. gonorrhoeae* multi-antigen sequence typing (NG-MAST), and multilocus sequence typing (MLST).

***porB*-based DNA Sequence Typing:** *porB*-based DNA sequence analysis is based on DNA sequence analysis of either full, or an extended length. of *porB*, that comprises most polymorphic segments, or the full length (up to nearly 1,000 bp) of the gene (Vicsidi et al 2000; Unemo et al 2002; Liao et al 2008; Unemo & Dillon 2011). This method has high discriminatory power when full-length or partial *porB* sequences are used (Unemo & Dillon 2011; Liao et al 2008). The cost for using this method is low as compared to other DNA sequence-based methods used and two DNA sequencing reactions of the amplicon are required (Hunter & Gaston 1988; Dillon et al 1993; van Looveren et al 1999). However, international standardization of the reference strain for full- or extended-length *porB* sequence-based methods and an appropriate database for these *porB* sequences, to permit international comparison, is lacking (Unemo & Dillon 2011).

***Neisseria gonorrhoeae* Multi-Antigen Sequence Typing:** NG-MAST involves analysis of variable internal fragments of two highly polymorphic loci of *N. gonorrhoeae*: *porB* (490 bp) and *tbpB* (390 bp), which encodes subunit B of the transferrin binding protein (Martin et al 2004). The combination of two hyper-variable loci ensures that the NG-MAST method has a very high discriminatory power. NG-MAST has been widely used because it is easy to perform and the availability of a public database (http://www.ng-mast.net) for comparing sequence types of *N. gonorrhoeae* isolates from different laboratories and geographical areas around the world. NG-MAST is most often used with cultured specimens, and the method must be optimized for potential use in all types of NAAT specimens (Whiley et al 2006; Unemo & Dillon 2011). Recently, Whiley et al (2009) demonstrated that NG-MAST can be applied to the non-cultured urogenital specimen but authors reported cross-reactions with commensal *Neisseria* for throat swabs.

Multiple Locus Sequence Typing: MLST evolved from the MLEE (De la Fuente & Vazquez 1992) and is based on DNA sequences of internal fragments of the alleles of seven or more chromosomal housekeeping genes (Maiden et al 1998; Bennett et al 2007). Housekeeping genes are conserved, slowly evolving, evolutionarily more neutral, and are distributed throughout the genome (Enright & Spratt 1999). For MLST analysis, different sequences for each locus are assigned divergent allele numbers, and the combination of alleles at the seven loci defines an allelic profile (Enright & Spratt 1999; Unemo & Dillon 2011). The MLST scheme for *N. gonorrhoeae* was originally adapted from MLST for *N. meningitides* [using *abcZ* (ATP-binding protein), *adk* (adenylate kinase), *aroE* (shikimate dehydrogenase), *gdh* (glucose-6-phosphate 1-dehydrogenase), *pdhC* (pyruvate dehydrogenase), *pgm* (phosphoglucomutase), *mtg* (myeloid translocation gene), *pilA* (regulatory protein PilA), *pip* (proline iminopeptidase), *ppk* (polyphosphate kinase) and *serC* (3-phosphoserine aminotransferase)] (Viscidi & Demma 2003; Maiden et al 1998). A slight modification of the "meningitidis" scheme with the addition of one gene [*abcZ, adk, aroE, gdh, pdhC, pgm, fumC* (fumarate hydratase class II)] has been used recently for molecular epidemiology of *N. gonorrhoeae* (Bennett et al 2007). A similar MLST scheme targeting seven loci [*gdh*, *fumC*, *pilA*, *serC*, *pyrD* (dihydroorotate dehydrogenase), *gnd* (6-phosphogluconate dehydrogenase) and *glnA* (glutamine synthetase)] has been used in studying gonococcal population dynamics (Viscidi & Demma 2003; Tazi et al 2010). MLST is

most useful for studying bacterial genomic evolution in bacterial populations (Viscidi & Demma 2003) since the house-keeping genes are under neutral selection and evolve slowly (Pérez-Losada et al 2005).

The main advantage of MLST over other existing *N. gonorrhoeae* typing schemes is that concatenated sequences (sequences from different genes linked in series) of the housekeeping genes (MLST) retain the phylogenetic uniqueness and genetic stability of gonococcal strains. Thus, MLST can help identify strains over a longer period of time, compared to DNA sequences of hyper-variable genes such as *porB* and *tbpB,* which can be subjected to frequent mutations and recombination, thereby, leading to a failure to identify the predominant related strains of *N. gonorrhoeae* (Vidovic et al 2011). MLST appears to be a more suitable method for long term epidemiological investigations to monitor the spread and distribution of gonococcal isolates (Vidovic et al 2011).

Lip-based DNA Sequence Typing: Lip-typing (Trees et al 2000) characterizes the number and sequences of repeats (encoding a five amino acid sequence, AAEAP) in the gene encoding an outer membrane lipoprotein (Woods et al 1989). By PCR-amplifying *lip* and subsequently differentiating the sizes of PCR products, the number of the repeat-coding sequences can be predicted. DNA sequence analysis of the amplicons further differentiates *N. gonorrhoeae* strains with the same number of repeats, thus identifying Lip subtyping patterns (Trees et al 2000).

Whole Genome Sequencing of *N. gonorrhoeae*: Bacterial strains can be differentiated through whole genome sequencing to investigate strain lineages and their transmission patterns (Harris et al 2010). This method can provide better discrimination compared to existing typing schemes and data generated from whole genome sequencing can be applied for short or long term epidemiological analysis. Comparative genome analysis for differentiating *N. gonorrhoeae* strains can be useful in the investigation of evolution of gonococci especially antimicrobial resistant strains and their determinants (Unemo & Dillon 2011). *N. gonorrhoeae* strains FA1090 (GenBank Accession #AE004969), NCCP11945 (GenBank Accession # CP001050) and TCDC-NG08107 (GenBank Accession # NC_017511) have been completely sequenced (Chung et al 2008; Chen et al 2011). The genome of *N. gonorrhoeae* FA1090 is 2.15 million nucleotides

(Mbp) in length with a GC content of 52.0%. There are 2002 protein-coding sequences and 67 structural RNA-coding sequences in the *N. gonorrhoeae* FA1090 genome. Sequencing of the *N. gonorrhoeae* NCCP11945 genome was completed in July 2008 (Chung et al 2008); it contains 2.232023 Mbps with a GC content of 52.0%. There are 2662 protein-coding sequences and 67 structural RNA-coding sequences in the *N. gonorrhoeae* NCCP11945 genome. In addition to these complete *N. gonorrhoeae* genomes, shotgun sequences of 16 *N. gonorrhoeae* strains are available from National Cenre for Biotechnology Information (NCBI. http://www.ncbi.nlm.nih.gov/nuccore)

Four *N. gonorrhoeae* plasmids have been completely sequenced, pJD4 (Pagotto et al 2000), pSJ5.2 (Scharbaai-Vázquez et al 2007), pCmGFP (GenBank Accession #FJ172221), pNGK (GenBank Accession #CP001051) and pEM1 (Muller et al 2011). These plasmids are 7.4 (pJD4), 5.16 (pSJ5.2), 6.1 (pCmGFP), 4.2 (pNGK) and 4.8 kbp (pEM1) long, respectively. The GC contents were variable, ranging from 38% to 51%.

1.3.2 Molecular Epidemiology of Gonorrhea

Epidemiology identifies factors that determine disease distribution in time and place, as well as factors that determine disease transmission, manifestation, and progression. The term "molecular epidemiology" was coined by Kilbourne in 1973 (Kilbourne 1973). Molecular epidemiology involves the use of the techniques of molecular biology to characterize nucleic acid or amino acid-based content, and "Epidemiology," the study of the distribution and determinants of disease (Foxman & Riley 2001). Riley (2004) defined molecular epidemiology of infectious diseases as "the study of the distribution and determinants of infectious diseases that utilize molecular biology methods." Molecular epidemiology helps to elucidate observations made in the biological world to formulate new concepts and make predictions. Molecular epidemiology determines the dynamics of disease transmission and identifies risk factors including genetic determinants.

Molecular techniques help to stratify and to refine data by providing more sensitive and specific measurements that facilitate epidemiologic analyses. This can, include disease

surveillance; outbreak investigations; identification of transmission patterns and risk factors among apparently disparate cases; characterization host-pathogen interactions; detecting uncultivatable organisms; providing clues for possible infectious causes of communicable and non-communicable diseases; and, providing better understanding of disease pathogenesis at the molecular level (Foxman & Riley 2001). *porB*-based DNA sequence analysis, NG-MAST and MLST have been used for studying the molecular epidemiology of *N. gonorrhoeae* (Unemo and Dillon 2011).

Gene markers for *porB* typing and NG-MAST evolve rapidly, owing to immune selection, which makes these typing methods virtually inapplicable in long-term epidemiological studies. The limitation of the NG-MAST and *porB* typing methods was overcome by introducing MLST (Maiden et al 1998). Unlike NG-MAST or *porB* typing methods, MLST provides clonal stability, while retaining a high-resolution power (Klint et al 2007; Meats et al 2003; Ilina et al 2010; Vidovic et al 2011). MLST schemes have been used to study gonococcal population structure, and emergence and evolution of antimicrobial resistance in *N. gonorrhoeae* (Viscidi & Demma 2003; Pe´rez-Losada et al 2005, 2007a, b; Bennett et al 2007; Tazi et al 2010, Ilina et al 2010; Mavroidi et al 2011; Vidovic et al 2011).

1.4 Population Dynamics and Emerging AMR Mechanisms of *Neisseria gonorrhoeae*: Saskatchewan as a Model

N. gonorroheae isolates collected from different clinics and hospitals across the province are subjected to antimicrobial susceptibility testing at the Saskatchewan Disease Control Laboratory (SDCL; Regina, SK) yet very limited information is available on the status of AMR in *N. gonorroheae* isolates from SK (PHAC 2011; Dillon 1990). This is primarily because fewer and fewer cases are diagnosed with cultures compared to NAATs. In a national study conducted in 1988/89, low prevalence of resistance to penicillin (0-0.9%) and tetracycline (0-2.7%) was recorded in SK (Dillon 1990). The treatment guidelines for gonorrhea in Canada have been changed since 1988/89 and no study has been conducted to determine the status of *N. gonorrhoeae* AMR in SK after 1988/89 (PHAC 2006; 2011c). Therefore, determination of the status of AMR in *N. gonorroheae* isolates from SK is relevant. No studies have been conducted

to determine the mechanisms of antimicrobial resistance in gonococcal isolates from SK. Reduced susceptibilities to extended spectrum cephalosporins and treatment failure with cefixime have been reported in Canada (Allen et al 2011, 2013; Martin et al 2012). It is worthwhile to explore AMR mechanisms in *N. gonorroheae* isolates from SK especially for extended spectrum cephalosporins.

Molecular epidemiological investigations conducted on *N. gonorroheae* isolates from the province are limited to a study conducted in 1988/89 determining the distribution of the auxotypes (A) and serovars (S) and A/S classes in the province (Dillon 1990). The most prevalent auxotypes in the province were; NR (59.7%), proline-uracil-hypoxanthine requiring (PUH, 12.1%) and proline-citruline-uracil requiring (PCU, 8.1%). The most prevalent (93%) serovars included; IB-3 (52.9%), IB-1 (19.2%), IB-2 (11.4%), and IA-2 (9.4). The majority (61.7%) of isolates were A/S classes NR/IB-3 (46.9%), PUH/IB-1 (8.6%) and PCU/IB-2 (6.2%). The prevalence of predominant A/S classes detected in this study was different from those for the rest of Canada (Dillon 1990).

No molecular epidemiological studies were conducted in province using DNA-based typing methods such as *porB* typing, NG-MAST and MLST. Use of these DNA-based typing methods will provide higher levels of discrimination and a better understanding of distribution and transmission of *N. gonorroheae* strains, emergence and evolution of AMR and for developing improved public health control measures, and preventive interventions. These would include contact tracing, identification of core groups, correlation with risk behaviors, and use of effective antimicrobial treatment in the province. The use of DNA based typing methods will help in developing baseline data on *N. gonorroheae* STs in SK. Based on such database, *N. gonorroheae* strain distribution, new strains and epidemiological parameters, and novel associations of antibiograms with specific strains/STs observed in SK can be compared internationally.

1.5 Hypothesis and Objectives

This study focuses on the investigation of the prevalence of antimicrobial resistance, mechanisms of antimicrobial resistance and the molecular epidemiology of *N. gonorrhoeae* isolated in the province of SK, Canada between 2003 and 2011.

Hypotheses:

1. Trends of antimicrobial resistance of *N. gonorrhoeae* in SK can differ temporally and geographically from Canada and worldwide, since isolates with raised MICs and decreased susceptibility to extended spectrum cephalosporins are emerging.
2. Specific genetic variations in various loci are associated with gonococcal antimicrobial resistance and will reflect a unique distribution in SK. These can be observed in targeted genes i.e. *penA, mtrR, porB,* and *ponA* for penicillin and extended spectrum cephalosporins resistance, *gyrA* and *parC* for quinolone resistance and *mtrR,* 23SrRNA and *erm* for azithromycin.
3. DNA-based typing schemes, based on unique AMR profiles can be used to determine whether *N. gonorrhoeae* strains from SK differ from gonococcal STs identified in other Canadian studies. Temporal and geographical differences will be observed between gonococcal STs in SK. Specific STs will be associated with specific resistance phenotypes or mutation patterns in potential resistance determinants in gonococcal isolates from SK .

Objectives:

The objectives of the present research are:

1. To ascertain the prevalence, patterns and trends of AMR in *N. gonorrhoeae* isolates in SK by:
   a) determining and analyzing antimicrobial susceptibility profiles to cefixime, ceftriaxone, azithromycin, spectinomycin, ciprofloxacin, penicillin and tetracycline or isolates collected from SK (2003-2011).

b) comparing SK trends to national and international data

2. Emerging AMR mechanisms will be ascertained by amplifying and analyzing DNA sequences of:
   a) *penA*, *mtrR*, *porB* and *ponA* for reduced susceptibility to extended spectrum cephalosporins and penicillin resistance.
   b) *gyrA* and *parC* quinolone resistance determinant regions (QRDR) and *mtrR* for ciprofloxacin resistance.
   c) *mtrR*, 23S rRNA alleles and *erm* encoding rRNA methylase enzymes for azithromycin resistance.
3. Investigating the distribution of *N. gonorrhoeae* STs in SK by molecular typing methods, specially ascertaining:
   a) strain distribution based on *porB* DNA sequence analysis, NG-MAST and MLST,
   b) phylogenetic relationships between STs,
   c) evolution of AMR phenotypes by MLST analysis,
   d) associations between specific STs and AMR phenotypes and genotypes.

CHAPTER TWO

MATERIALS AND METHODS

2.1 Collection and Identification of *Neisseria gonorrhoeae* Isolates

Cultures of *N. gonorrhoeae* (n=427) were consecutively collected at the Saskatchewan Disease Control Laboratory (SDCL), Regina, SK between 2003 and 2011. None of the isolates tested were duplicate specimens from the same patient at the same time period. Primary isolations of *N. gonorrhoeae* were made at the SDCL on Thayer Martin medium (OXOID, ON, Canada) containing 1% IsovitaleX **(**Becton Dickinson, ON, Canada) (Knapp 1988). The isolates were identified as *N. gonorrhoeae* at the SDCL. 320 of these isolates were shipped in Amies transport medium (Becton Dickinson, Mississagua, ON, Canada) to the University of Saskatchewan. They were stored at –80^{o}C in Brain Heart Infusion medium (Difco BD Biosciences, Oakville, ON, Canada) containing 20% glycerol. Frozen *N. gonorrhoeae* isolates were streaked onto GC Agar Medium Base (GCMB, Difco BD Biosciences, Oakville, ON, Canada) supplemented with 1% modified Kellogg's supplement (GCMBK, Kellogg et al 1963) and incubated at 35°C with 5-7% CO_2 in a humid environment for 20-24 hours (Knapp 1988; CLSI 2009). Isolates were reconfirmed as *N. gonorrhoeae* using the oxidase test and Gram staining (WHO 2003). Information such as city of isolation, age and sex of the patient was available for each isolate.

2.2 Antimicrobial Susceptibility

2.2.1 Determination of Minimal Inhibitory Concentrations

The antimicrobial susceptibility of the *N. gonorrhoeae* isolates was determined using the agar dilution method of the Clinical and Laboratory Standards Institute (CLSI 2009).World Health Organization (WHO) reference strains B, C, F and K and ATCC 49226 (Table 2.1) were used as reference strains for antimicrobial susceptibility testing (CLSI 2009; WHO-WPR 1997; Unemo et al 2009). The antimicrobial susceptibility of 427 *N. gonorrhoeae* isolates was determined to penicillin (0.002–128.0 mg/L), tetracycline (0.06–256.0 mg/L), ciprofloxacin (0.001–64.0 mg/L), azithromycin (0.016–32.0 mg/L), cefixime (0.00025-2.0 mg/L), ceftriaxone (0.000125–2.0 mg/L) and spectinomycin (4.0–256.0 mg/L) (CLSI 2009). Of the minimum inhibitory concentration (MIC) data in this study, 320 *N. gonorrhoeae* isolates (2003-2008) were tested by me including susceptibility to azithromycin and data for a further 107 isolates (2009-

Table 2.1 Strains and plasmid used in this study as reference strains and positive controls

Strain	Phenotype[f]	Reference	Purpose	Genes/Plasmids amplified	Source
WHO B	Pen^{I}, Tet^{I}	WHO 1997	Antimicrobial susceptibility testing		
WHO C	Pen^{I}, Tet^{I}	WHO 1997	Antimicrobial susceptibility testing		
WHO F	Tet^{I}	WHO 1997	Antimicrobial susceptibility testing		
WHO G	TRNG	Unemo et al 2009	DNA amplification	Dutch type *tetM* plasmid	
ATCC 49226	Pen^{I}, Tet^{I}, Ery^{R}	CLSI 2009	Antimicrobial susceptibility testing		
WHO K[a]	CMRNG, Cip^{R}, Cef^{NS}, Cro^{NS}	Unemo et al 2009	Antimicrobial susceptibility testing, transformations, DNA amplification	*penA*, *ponA*, *mtrR*, *gyrA*, *parC*, *porB*	NML[g], Winnipeg, Canada
WHO L[b]	CMRNG, Cip^{R}, Cef^{NS}, Cro^{NS}	Unemo et al 2009	DNA amplification	*penA*, *parC*,	
WHO M[c]	PPNG, Cip^{R}	Unemo et al 2009	DNA amplification	*gyrA*,	
WHO N[d]	PPNG, TRNG, Cip^{R}	Unemo et al 2009	DNA amplification	*mtrR*	
WHO P[e]	Ery^{R}, Azi^{R}	Unemo et al 2009	DNA amplification	*mtrR*	
FA19	Sucesptible	Zarantonelli et al 1999	Transformations, DNA amplification	recipient strain, wild type *penA*	Dr. W Shafer, Emory University, USA
Ng 9	Pen^{I}, Tet^{R}	This study	Transformations, DNA amplification	*penA*, non-mosaic pattern IX	
Ng 22	Pen^{I}, Tet^{R}	This study	Transformations, DNA amplification	*penA*, non-mosaic pattern XXII	SDCL[h], Regina, Canada
Ng 34	TRNG, Cip^{R}, Cef^{NS}, Cro^{NS}	This study	Transformations, DNA amplification	*penA*, mosaic pattern XXXIV	
Staphylococcus aureus RN1389	NA	Ng et al 2002	DNA amplification	*ermA*	
S. pyogenes AC1	NA	Ng et al 2002	DNA amplification	*ermB*	Dr. I Martin, NML, Winnipeg, Canada
S. aureus RN4220	NA	Ng et al 2002	DNA amplification	*ermC*	
E. coli V831	NA	Ng et al 2002	DNA amplification	*ermF*	
pJD4		Dillon & Yeung 1989	DNA amplification	Asian β-lactamase	Dr. JR Dillon, University of Saskatchewan, Canada
pJD5		Dillon & Yeung 1989	DNA amplification	African β-lactamase	
pJD7		Dillon & Yeung 1989	DNA amplification	Toronto β-lactamase	

a: *penA*, mosaic pattern X with I312M, V316T& G545S; *ponA*, L421P; *mtrR*, deletion of A (promoter) & G45D in coding sequence; *gyrA*, S91F; *parC*,S87R &S88P; *porB*, G120K & A121D; b: *penA*, D345a & A501V; *parC*, D86N &S88P; c: *gyrA*,S91F & D95N; d: *mtrR*, truncated mtrR; e: *mtrR*, A to C substitution (promoter) & insertion of T at bp 60; f: Phenotypes-Pen^{I}-penicillin intermediate susceptibility, Tet^{I}-tetracycline intermediate susceptibility, TRNG-tetracycline resistant *N. gonorrhoeae*, Ery^{R}-erythromycin resistant, CMRNG; chromosomally mediated resistant *N. gonorrhoeae*, Cip^{R}-ciprofloxacin resistance, Cef^{NS-} not susceptible to cefixime, Cro^{NS}-not susceptible to ceftriaxone, PPNG-penicillinase producing *N. gonorrhoeae*, Ery^{R}-erythromycin resistance, Azi^{R}-azithromycin resistance; g: NML- National Microbiological Laboratory; h: SDCL- Saskatchewan Disease Control Laboratory.

2011) was obtained from the SDCL. For testing performed in Saskatoon, all antimicrobial agents were purchased from Sigma Aldrich (Oakville ON, Canada). Minimum Inhibitory Concentrations (MICs) were determined in duplicate on GCMBK agar, and included reference strains for each time performed. Antimicrobial susceptibly/resistance criteria (Table 2.2) were those of CLSI (CLSI 2009). For azithromycin, since no breakpoints have been established by the CLSI, interpretative criteria were based on the Gonococcal Isolate Surveillance Project (GISP), USA (CDC 2010b). Gonococcal inocula were prepared by suspending an overnight culture grown on GCMBK plates in Muller-Hinton broth (Difco, BD Biosciences, Oakville, ON, Canada) to a 0.5 McFarland turbidity standard (Remel, Lenexa KS, USA), which equilibrates to 10^8 colony forming units (CFU) per milliliter. This suspension was diluted 1:10 with Muller-Hinton broth, resulting in a cell density of ~10^7 CFU/mL. Approximately 2 μL of the cell suspension containing 200 CFU was delivered onto antibiotic- containing and control GCMBK plates using a multispot Steers inoculator (Steers et al 1959) and incubated for 18-24 hours at 35^{o}C with 5-7% CO_2 in a humid environment. The MIC was considered to be the lowest concentration of antibiotic producing complete inhibition of growth (CLSI 2009). As per the CDC, the alert values for cefixime and ceftriaxone are MICs 0.125 mg/L and 0.25 mg/L and are defined as elevated MICs (CDC 2012). β-lactamase production was determined using the chromogenic nirocefin test (Calbiochem, EMD Chemicals Inc., Darmstadt, Germany). All isolates with penicillin MICs ≥ 2.0 mg/L were tested for β-lactamase production.

The resistance phenotypes classified in this study included: PPNG (β-lactamase positive);TRNG (tetracycline MIC ≥16 mg/L and β-lactamase negative); PP/TRNG (β-lactamase positive and tetracycline MIC ≥16 mg/L); Pen^R (non PPNG; penicillin MIC ≥ 2.0 mg/L);CMTR (non-PPNG; non-TRNG, penicillin MIC < 2.0 mg/L and tetracycline MIC ≥ 2.0-8.0 mg/L); Cip^R (ciprofloxacin MIC ≥ 1.0 mg/L); Azi^R (azithromycin MIC ≥ 2.0mg/L); Spec^R (spectinomycin MIC ≥ 128.0 mg/L); CMPR (non-PPNG, tetracycline MIC < 2.0 mg/L and penicillin MIC ≥2.0 mg/L);,and, CMRNG (non-PPNG, non-TRNG, penicillin MIC ≥ 2.0 mg/L and tetracycline MIC ≥ 2.0 mg/L). An isolate can exhibit more than one resistance phenotype. For example, the phenotype of CMTR/Azi^R/PPNG indicates an isolate of *N. gonorrhoeae* with chromosomal resistance to tetracycline, azithromycin, which is also β-lactamase positive.

Table 2.2 Determination of minimal inhibitory concentrations and interpretation criteria for *N. gonorrhoeae*[a]

Antimicrobial agent	Antibiotic concentration Testing range (mg/L)	Interpretative criteria (mg/L)[a]		
		S	I	R
Penicillin	0.002 – 128.0	≤ 0.06	0.12 – 1.0	≥ 2.0
Tetracycline	0.06 – 256.0	≤ 0.25	0.5 – 1.0	≥ 2.0
Ciprofloxacin	0.001 – 64.0	≤ 0.06	0.12 – 0.5	≥ 1.0
Azithromycin[b]	0.016 – 32.0	≤ 1.0	ND[d]	≥ 2.0
Cefixime	0.00025 – 2.0	≤ 0.25[c]	ND	ND
Ceftriaxone	0.00125 – 2.0	≤ 0.25[c]	ND	ND
Spectinomycin	4.0 – 256.0	≤ 32.0	64.0	≥ 128.0

a: Interpretative criteria for MIC classification were those described by the CLSI (CLSI 2009). MICs are classified into susceptible (S), intermediate (I) and resistant (R) for each antimicrobial agent.
b: Interpretative criteria for azithromycin MIC classification are not described by CLSI, therefore standards (alert values) followed by Gonococcal isolate surveillance project (GISP), USA were used (CDC 2010b).
c: Alert values for cefixime and ceftriaxone are MICs 0.125 mg/L and 0.25 mg/L (CDC 2012).
d: Break points are not defined.

For cefixime and ceftriaxone, the susceptible breakpoint is defined as having MICs ≤ 0.25 mg/L (CLSI 2009; CDC 2010b). Breakpoints for intermediate-levels of susceptibility and resistance have not been defined for cefixime and ceftriaxone. The CLSI defines reduced susceptibility to cefixime and ceftriaxone as having MICs > 0.25 mg/L, whereas the European Committee on Antimicrobial Susceptibility Testing (EUCAST) defines reduced susceptibility to cefixime MIC > 0.25 mg/L and ceftriaxone MIC > 0.125 mg/L (Cole et al 2010). In the USA, alert values for cefixime and ceftriaxone reduced susceptibility are MICs 0.125 mg/L and 0.25 mg/L (CDC 2012).

2.3 Molecular Mechanisms of Antimicrobial Resistance in *N. gonorrhoeae*

2.3.1 Chromosomal DNA Isolation

DNA isolation was performed using overnight *N. gonorrhoeae* growth from approximately one half of a GCMBK plate and suspendeding it in 1 mL of 0.9% saline. After centrifugation at 10,000 X g for 1 min the supernatant was removed and genomic DNA was extracted using a Qiagen DNeasy Tissue Kit (Qiagen Inc., Valencia, California) according to the manufacturer's instructions. DNA was eluted in sterile distilled water and stored at -20^{o}C. The chromosomal DNA of WHO reference strains K, L, M, N and P used as positive controls for various resistance genotypes (Unemo et al 2009) (Table 2.1).

2.3.2 Amplification of *penA*, *mtrR*, *porB*, *ponA*, *gyrA*, *parC*, 23SrRNA and *erm* by the Polymerase Chain Reaction

The DNA sequences of *penA*, *mtrR*, *ponA1* and *porB* were analyzed for 146 [2003 (n=24, 40%), 2004 (n=37, 62%), 2005 (n=29, 55%), 2006 (n=18, 33%), 2007 (n=22, 42%) and 2008 (n=16, 39%)] isolates to determine the emerging molecular mechanisms of extended spectrum cephalosporin susceptibility. This subset of isolates included all the Pen^{R} (n=15), Cip^{R} (n=5), Azi^{R} (n=2) and 50% (n=90) of Tet^{R} isolates observed from 2003 through 2008 in this study. No isolates with" reduced susceptibility" (i.e. CLSI value MIC≥0.25 mg/L) to cefixime and ceftriaxone were observed in this study. In order to ascertain the emerging mechanisms of reduced susceptibility or resistance to extended spectrum cephalosporins these isolates were

divided into two susceptibility groups: isolates with MIC 0.0005-0.016 mg/L (cefixime & ceftriaxone susceptibility group 1; n=123) and isolates with MICs 0.03-0.06 mg/L (cefixime & ceftriaxone susceptibility group 2; n=23). 23S rRNA and *mtrR* DNA sequences were analyzed in 52 isolates for azithromycin resistance. Presence of *erm* (rRNA methylase genes) was also determined in these isolates. *gyrA*, *parC* and *mtrR* DNA sequences for 41 *N. gonorrhoeae* isolates were analyzed for ciprofloxacin resistance. All the resistant isolates detected in this study were analyzed for the molecular mechanisms of resistance.

Primers used for polymerase chain reaction (PCR) and DNA sequencing reactions are listed in Table 2.3. Primers were purchased from Invitrogen Canada (Burlington, ON). Primer stock solutions were prepared by dissolving individual primers in sterilized distilled water at a concentration of 100 μM. Antibiotic resistance genes were amplified using the following primer pairs: penA-F/penA-R for *penA* (Ochiai et al 2007; Lee et al 2010); mtrR-F/mtrR-R for the *mtrR* promoter and coding sequences (Xia et al 2000); porB-F/porB-R for *por*B (Martin et al 2004; Unemo et al 2003); ponA-F/ponA-R for *ponA* (Ropp et al 1997, 2002); gyrA-F/gyrA-R for *gyrA*; and, parC-F/parC-R for *parC* (Belland et al 1994). The DNA sequences of an internal segment of the *porB* gene are also used for *N. gonorrhoeae* Multi-Antigen Sequence Typing (NG-MAST) (Martin et al 2004). Primer pair tbpB-F/tbpB-R was used to amplify an internal segment of *tbpB* for NG-MAST analysis (Martin et al 2004).

Four alleles of the 23S rRNA gene were amplified individually using primer pairs gonrRNA-F/23S rRNAR-allele1 for allele 1; gonrRNA-F/23S rRNAR-allele2 for allele 2; gonrRNA-F/23S rRNAR-allele3 for allele 3; gonrRNA-F/23S rRNAR-allele4 for allele 4; and, gonrRNA-F/gonrRNA-R2 for the 23S rRNA peptidyltransferase loop (Ng et al 2002). Amplification reactions for the detection of rRNA methylase genes, *ermA*, *ermB*, *ermC* and *ermF* conferring resistance to macrolides were performed using primer pairs; A_F/A_R for ermA, B_F/B_R for *ermB*, and C_F/C_R for *ermC* and F_1/F_2 for *ermF* (Chung et al 1999).

Table 2.3 Primers used for PCR and DNA sequencing reactions for *penA*, *mtrR*, *porB*, *ponA*, *gyrA*, *parC*, 23SrRNA and *erm*

Primer Name	Sequence	Locus and GenBank accession number	Amplified and Sequenced regions	References
porB-F porB-R	CCGGCCTGCTTAAATTTCTTA TATTAGAATTTGTGGCGCAG	*porB*, J03017	nt[a]72-933, coding for AA[b] 25-311	Unemo et al 2003, Martin et al 2004
tbpB-F tbpB-R	CGTTGTCGGCAGCGCGAAAAC TTCATCGGTGCGCTCGCCTTG	*tbpB*, 2286066	nt1098-1686	Martin et al 2004
penA-F penA-R	CGATATGATCGAACCTGG ACAATCTCGTTGATACTCG	*penA*, X07468	nt1017-1725, coding AA340-575	Ochiai et al 2007, Lee et al 2010
mtrR-F mtrR-R	AACAGGCATTCTTATTTCAG TTAGAAGAATGCTTTGTGTC	*mtrR*, Z25796	nt1-120, nt 1-630 coding sequences of full-length MtrR (AA1-210)	Xia et al 2000
ponA-F ponA-R	CGCGGTGCGGAAAACTATATCGAT AGCCCGGATCGGTTACCATACGTT	*ponA*, U72876	nt987-1950, coding for AA330-650	Ropp et al 1997, 2002
gonrRNA-F 23S rRNAR-allele1	ACGAATGGCGTAACGATGGCCACA TCAGAATGCCACAGCTTACAAACT	23S rRNA allele1, AF450074	nt1-597	
gonrRNA-F 23S rRNAR-allele2	ACGAATGGCGTAACGATGGCCACA GCGACCATACCAAACACCCACAGG	23S rRNA allele2, AF450076	nt1-610	
gonrRNA-F 23S rRNAR-allele3	ACGAATGGCGTAACGATGGCCACA GATCCCGTTGCAGTGAAGAAAGTC	23 SrRNA allele3, AF450078	nt1-606	Ng et al 2002
gonrRNA-F 23S rRNAR-allele4	ACGAATGGCGTAACGATGGCCACA AACAGACTTACTATCCCATTCAGC	23S rRNA allele4, AF450080	nt1-617	
gonrRNA-F gonrRNA-R2	ACGAATGGCGTAACGATGGCCACA TTCGTCCACTCCGGTCCTCTCGTA	23S rRNA peptidyltransferase loop		
A_F A_R	CTTCGATAGTTTATTAATATTAG TCTAAAAAGCATGTAAAAGAA	*ermA*		Roberts et al 1999; Chung et al 1999

a: nt- nucleotide positions; b: AA- amino acid position

Table 2.3 continued……..

Primer Name	Sequence	Locus and GenBank accession number	Amplified and Sequenced regions	References
B_F	AGTAACGGTACTTAAATTGTTTAC	*ermB*		
B_R	GAAAAGGTACTCAACCAAATA			
C_F	GCTAATATTGTTTAAATCGTCAAT	*ermC*		Roberts et al 1999; Chung et al 1999
C_R	TCAAAACATAATATAGATAAA			
F_1	CGGGTCAGCACTTTACTATTG	*ermF*		
F_2	GGACC TACCTCATAGACAAG			
gyrA-F	ACTGTACGCGATGCACGAGC	*gyrA*, U08817	nt[a]195-432, coding for AA66-144	Belland et al 1994
gyrA-R	TCTGCCAGCATTTCATGTGAG			
parC-F	GTTTCAGACGGCCAAAAGCC	*parC*, U08907	nt120-399, coding AA41-133	Belland et al 1994
parC-R	GGCATAAAATCCACCGTCCCC			
JDA	TACTCAATCGGTAATTGGCTTC	β-lactamase		Dillon et al 1999
JDB	CCATATCACCGTCGGTACTG			
RM4	CCAAATCCTTTCTGGGCT	*tetM*		Xia et al 1995
G1	ATCACTCAGTTTAAT			
penF	CGGGCAATACCTTTATGGTGGAAC	*penA*, AB546858	nt[a]1-1749, coding for AA[b] 1-583	Ohnishi et al 2011b
penR	ACAACGGCGGCGGGGATATAAC			
penA_SF1	CAAAGATAGAAGCAGCCTG			
penA_SF2	GATATTGACGGCAAAGGTC			
penA_SF3	CTTTGGATGTGCGCGGC			
penA_SR1	GCCGTCGGTATATTCGC			
penA_SR2	CCAAAGGGGTTAACTTGC			
penA_SR3	TTCTCAACAAACCTGCAG			
penA_SR4	CTTTGCCGTTTTGCGGGG			

a: nt- nucleotide position

The PCR mixture (50 µL) for all reactions contained 2 µL of genomic DNA, 2.5 U of dreamTaq DNA polymerase (Fermentas, Burlington, ON, Canada L7N 3N4), 1 x PCR buffer with 1.5 mM $MgCl_2$, 0.25 mM dNTPs and 0.5 µM of each primer. Amplification (MyCycler; Applied Biosystems, Streetsville, Ontario, Canada) was performed as follows: an initial denaturing step at 94^{o}C for 4 min, followed by 30 sequential cycles of 94^{o}C for 1 min, 55^{o}C for 45 s, 72^{o}C for 45 s (for *tbpB, gyrA* and *parC*) or 1 min (for *porB, mtrR, penA* and *ponA*), and a final extension phase at 72^{o}C for 10 min (Liao et al 2008, 2011).

23S rRNA was amplified using the following conditions: 1 min of denaturation at 94°C, 1.5 min at 66°C (for alleles 2 and 3) or 68°C (for alleles 1 and 4) for annealing, and 2.5 min at 72°C for elongation for 30 cycles (Ng et al 2003). The amplicons obtained were then used as templates in a second PCR using gonrRNA-F and gonrRNA-R2 primers to amplify the peptidyltransferase loop for sequence determination (Ng et al 2003). The conditions for this PCR reaction were 94°C at 1 min for denaturing, 59°C for 1 min for annealing, and 72°C at 1 min for elongation for 35 cycles (Ng et al 2003).

Amplification conditions for *ermB* and *ermF* were as follows (Roberts et al 1999; Chung et al, 1999): denaturing at 94°C for 30 s; annealing at 50°C for 30 s; and elongation at 72°C for 2 min. The cycle was repeated 35 times. *ermA* amplification consisted of denaturing at 94°C for 30 s, annealing at 48°C for 1 min, and elongation at 72°C for 2 min. Amplification of *ermC* consisted of denaturing at 94°C for 30 s, annealing at 43°C for 1 min, and elongation at 72°C for 2 min. *Staphylococcus aureus* RN1389, *S. pyogenes* AC1, *S. aureus* RN4220 and *E. coli* V831 (Table 2.1; provided by Irene Martin, National Microbiology Laboratory, Winnipeg, Canada) were used as positive controls for PCR amplifications of *erm*A, *erm*B, *erm*C and *erm*F) (Roberts et al 1999; Chung et al 1999; Ng et al 2002).

Amplified DNA products were analyzed by agarose gel (1%) electrophoresis followed by ethidium bromide (0.5 µg/mL) staining and visualization under UV light (Universal Hood II, Biorad, Mississauga, ON, Canada) (Shambrook & Russel 2001). They were examined for purity and size as compared to DNA standards (1kb Plus DNA ladders, New England Biorad, Mississauga, ON, Canada). All PCR products were purified using the Qiagen PCR Purification

Kit according to the manufacturer's instructions (Qiagen, Mississauga, ON, Canada). DNA was eluted in 50 μL sterilized ddH_2O and stored at -20^oC for use in subsequent DNA sequencing analysis.

2.3.3 Determination of β lactamase and *tetM* Plasmid Type

β-lactamase producing plasmids were typed by PCR assay using the primer pair JDA/JDB ((Dillon et al 1999; Table 2.3). Purified DNA of pJD4 (Asian β-lactamase plasmid), pJD5 (African β-lactamase plasmid) and pJD7 (Toronto β-lactamase plasmid) (Table 2.1), available in the Dillon laboratory were positive controls and a β-lactamase negative (WHO G) isolate and sterilized distilled water were negative controls (Table 2.1; Dillon et al 1999; Unemo et al 2009). *tetM* plasmid types were determined for isolates with tetracycline MICs ≥ 16 mg/L by PCR using primer pairs RM4/G1 (Table 2.3; Xia et al 1995). Strain WHO G with Dutch type *tetM* plasmid was used as a TRNG positive control (Table 2.1; Unemo et al 2009). PCR conditions were the same as described in Section 2.3.2, except for an extension time of 5 min for β-lactamase and 2 min for *tetM* in the reaction cycles (Table 2.3; Xia et al 1995; Dillon et al 1999). Amplified DNA products were analyzed by and visualized as described in Section 2.3.2. The sizes of PCR products of pJD4, pJD5, and pJD7 were 4.9 kb, 3.1 kb, and 2.6 kb, respectively (Dillon et al 1999). The America-type *tetM*-containing plasmid has an amplicon size of 1.6 kb and the Dutch-type *tetM*-containing plasmid exhibits a fragment of 0.7 kb (Xia et al 1995).

2.3.4 DNA Sequencing

In total, 1540 DNA sequences of genes implicated in AMR of *N. gonorrhoeae* [*penA* (n=292); *mtrR* (n=292); *porB* (n=292); *ponA* (n=292); *gyrA* (n=82); *parC* (n=82) and, 23S RNA (n=208)] were amplified and analyzed. Primers used for DNA sequencing reactions of the targeted loci were the same as the primers used in PCR reactions except for 23SrRNA where primer pair gonrRNA-F/ gonrRNA-R2 (Table 2.3) was used. DNA sequences of both strands for each locus amplified were determined using an Applied Biosystems 3730x1 DNA Analyzer (Plant Biotechnology Institute, National Research Council of Canada, Saskatoon, SK). DNA sequences were verified and edited using online programs. Chromas Lite

(http://www.softpedia.com/get/Science-CAD/Chromas-Lite.shtml) was used to view and edit chromatograms. The ClustalW program (http://www.ebi.ac.uk/Tools/clustalw2/index.html) was used to align DNA sequences of both complementary strands (Larkin et al 2007). Aligned DNA sequences were viewed and edited using the Jalview program (Clamp et al 2004). A reference sequence for each locus was used for trimming DNA sequences (Table 2.4). Flanking sequences corresponding to the reference sequence were removed or trimmed. After removing gaps introduced during alignment, DNA sequences were output in a FASTA format. DNA sequences were copied and pasted in Notepad, creating DNA sequence databases for subsequent analysis.

Predicted amino acid sequences for each gene were obtained using the Proteomics and Sequence Tools (http://ca.expasy.org/, last accessed on December 12, 2012). To identify mutations, amino acid sequences were aligned with their respective wild type prototype reference sequences for which GenBank accession numbers and sequence lengths were shown in Table 2.4.

Amino acid sequence patterns of PBP2 (*penA*) were classified and numbered according to criteria used by others (Ito et al 2005; Whiley et al 2007a; Ohinishi et al 2011b). Mutations in *mtrR, porB, ponA1* and were classified as described previously (Warner et al 2008; Olesky et al 2002; Ropp et al 2002). Novel sequences or mutations identified in this study have been submitted to the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/WebSub/?tool=genbank).

A comprehensive data base on antimicrobial susceptibility, molecular mechanisms of resistance and STs has been generated on *N. gonorrhoeae* isolates analyzed during this study and is consistent with earlier studies conducted in Dillon laboratory (Yang et al 2006; Liao et al 2008, 2011).

Table 2.4 Reference DNA sequences used in this study[a]

Locus	Nucleotide position	GenBank accession number
*porB*1a (*N. gonorrhoeae* MS11, Extended-length	nt[c]72-983, gaps included	J03029
*porB*1b[a] (*N. gonorrhoeae* FA 1090, Extended-length)	nt[c]72-933, gaps included	J03017
porB (for NG-MAST[b])	Start at nt350 extended for 490 bp, gaps removed during alignment	M21289
tbpB[a]	Start at nt1098 extended for 390 bp, gaps removed during alignment	2286066
gyrA	nt220-360, QRDR[d] coding sequences	U08817
parC	nt210-390, QRDR coding sequences	U08907
mtrR (*N. gonorrhoeae* FA1090)	nt-1 to -120 promoter sequences and sequences coding for full-length MtrR (nt1-630)	Z25796
penA	nt1017-1725, coding for AA[e]340-575 of PBP2[f] (full-length PBP2 581 AAs)	M32091
ponA1	nt1050-1890 coding AA350-630 of PBP1 (full-length PBP1 798 AAs)	U72876
23S rRNA	nt1-611	AF450074-AF450081

a: *porB* (NG-MAST) and *tbpB* sequences are the prototypes used for NG-MAST typing (Martin et al 2004). All other DNA sequences are wild type prototypes and were used as reference sequences for the determination of mutations in different resistant determinants.

b: NG-MAST- *Neisseria gonorrhoeae* multi-antigen sequence typing.

c: nt-nucleotide positions.

d: AA-amino acid.

e: QRDR-quinolone resistance determinant region.

f: PBP- penicillin binding protein.

2.4 Population Dynamics of *Neisseria gonorrhoeae* in Saskatchewan by Analysis of *porB*, NG-MAST and MLST

The molecular epidemiology of isolates of *N. gonorrhoeae* from SK was determined using the *porB* sequence typing, *N. gonorrhoeae* Multi-Antigen Sequence Typing (NG-MAST) and multilocus sequence typing (MLST). *porB* sequence typing and NG-MAST was performed on 320 isoltes collected between 2003 and 2008. A subset of 193 isolates recovered between 2005 and 2008 were analyzed by MLST. In all, 3982 sequences [*porB* typing (n=640), NGMAST (n=640, for *tbpB*) and MLST (n=2702)] were amplified and analyzed to ascertain the population dynamics of *N. gonorrhoeae* in SK.

The *porB* DNA sequences determined in this study comprised a fragment (765bp, ≈77% of *porB* sequence) containing 7 polymorphic regions encoding the surface-exposed loops (Loops I - VII) and 6 conserved interspace regions (II - VII) (Liao et al 2008, 2009). The sequences of these regions were determined for 320 *N. gonorrhoeae* isolates collected between 2003 and 2008.). *porB* loci were amplified and sequenced (Table 2.3) as described earlier in Section 2.3.2 (Table 2.3). *N. gonorrhoeae porB*1a (GenBank #J03029), and *porB*1b (GenBank #J03017) (Table 2.4) were used as prototype to edit the amplified *porB* sequences. *porB* strains types were assigned numbers consistent with the typing criteria used in the Dillon laboratory (http://www.usask.ca/biology/dillon/) (Liao et al 2008, 2009).

The NG-MAST database and programs for assigning STs of *N. gonorrhoeae* isolates are available online (www.ng-mast.net, Martin et al 2004). *tbpB* and *porB* loci were amplified by PCR and amplicons were sequenced as described in Section 2.3.2 (Table 2.3). An allele type was assigned to either *porB* or *tbpB* of each isolate, and a ST was assigned to the isolate based on *porB* and *tbpB* types online (www.ng-mast.net). Previously identified alleles of *porB* and *tbpB*, and STs were obtained by interrogating the NG-MAST website. The unrecognized NG-MAST STs were identified as novel STs on the basis of new alleles and ST numbers.

193 gonococcal isolates (2005-2008) were typed by using multilocus sequence typing (MLST). Partial regions of 7 housekeeping genes *abcZ, adk, gdh, glnA, gnd, fumC* and *pyrD*

were amplified using primers listed in Table 2.5. PCR conditions included an initial 95^0C denaturation step for 4 min, followed by 30 cycles of: denaturation at 95^0C for 60 s, annealing at 55^0C for 30 s, amplification at 72^0C for 30 s and a final extension step at 72^0C for 10 min. Amplicons were purified and sequenced as described in Section 2.3.2 and 2.3.4. All sequences for each locus were compared and every unique sequence was assigned as a distinct allele using Sequence Output (www.mlst.net). Each unique combination of allele numbers was assigned as a different ST.

Grouping of *porB* and NG-MAST STs with less than 1% base pair difference: The alleles associated with most predominant *porB* and NG-MAST STs, were compared against all the alleles observed in this study for 320 isolates to determine similarity between different STs. It was done by multiple alignments using ClustalW, followed by individual pair wise alignment against most frequent alleles. All the *porB* STs with ⩾99% similarity (⩽7 bp difference for *porB*) were grouped together and such groups were named after predominant *porB* STs. NG-MAST STs were grouped as one NG-MAST group if one identical allele (*porB* and *tbpB*) was shared and showed ≥99% similarity in other allele (≤5bp difference for *porB* and ≤4bp for *tbpB*) (Chisholm et al 2013). The NG-MAST groups were named after predominant NG-MAST ST in each group.

2.4.1 Phylogenetic Analysis of Sequences Analyzed for *porB* Typing, NG-MAST and MLST

porB DNA sequences in FASTA format were converted into a NEXUS format using the ClustalX program (http://bips.u-strasbg.fr/fr/Documentation/ClustalX; Thompson et al 1997) for use in TreeView program. Branch nodal support was assessed via bootstrapping (1,000 pseudoreplicates). Trees were figured and viewed in Treeview (Page 1996; http://taxonomy.zoology.gla.ac.uk/rod/treeview.html).

MEGA4 was used to construct phylogenetic trees from concatenated sequences of the housekeeping genes amplified for MLST (Tamura et al 2007). The evolutionary distances were

Table 2.5 Primers used for PCR and DNA sequencing reaction in multilocus sequence typing

Locus	Putative function	Fragment amplified (bp)[a]	Primer name	Sequence (5′ to 3′)
abcZ	ATP-binding protein	498	abcZ F abcZ R	AATCGTTTATGTACCGCAGG GAGAACGAGCCGGGATAGGA
adk	Adenylate kinase	522	adk F adk R	CGTTCGGCATTCCGCAAATCTCT CGACTTTGATGTATTTCGGCGC
gdh	Glucose-6-phosphate 1-dehydrogenase	631	gdh F gdh R	ATGTTCGAGCCGCTGTGGAACAA CTTCAACGGCCTTGCCCAAATCC
glnA	Glutamine synthetase	537	gln F gln R	GTTTGAAAACGGACCGGCGTTTGA TTCCATTTGGCTGCCGGTACCGAC
gnd	Phosphogluconate dehydrogenase	549	gnd F gnd R	GAGAAAATCCTCGATACGGCAGGGCA GGTCGTATAGCCGTCCAAGAACGT
fumC	Fumarate hydratase class II	535	fumC F fumC R	GCCATCCCGAATACGCTGAAATAG CGATTTTGCGGTTTAACGCAGTAAC
pyrD	Dihydroorotate dehydrogenase	592	pyrD F pyrD R	GATGATGCCGTCCATTTCGACGGA TATAAAACTGATGGGTATGGATTTGC

a: bp-base pair

computed using the Maximum Composite Likelihood method (Tamura et al, 2004). The Neighbor-joining algorithm (Saitou and Nei 1987) was used to generate the initial tree.

To predict the ancestral profiles and clonal complexes, each genotype identified in this study was analyzed by eBURST (Feil et al 2004). eBURST groups were formed by the isolates with similar AMR phenotype. An eBURST group is defined as a collection of STs that are placed together according to the selected group definition. The STs within an eBURST group obtained with the most stringent (exclusive) group definition were closely related and belong to a single clonal complex. A clonal complex refers to a biologically meaningful cluster of STs that have diversified very recently from a common founder. Clonal complexes are defined as a cluster of STs in an eBURST diagram in which all STs are linked as single locus variances (SLVs) to at least one other ST. The primary founder of the clonal complex was predicted as the ST that has the largest number of SLVs in the group or clonal complex. Statistical confidence in each of the assigned primary founders was made by a bootstrap resampling (1,000) procedure. For an ST which was SLV of both primary and subgroup founders, the ST was assigned to the primary founder. When an ST was an SLV of two or more subgroup founders, then the ST was grouped with the largest subgroup.

For identification of clonal complexes, the most stringent default group definition was used, where all STs have to be single locus variants of at least one ST in the clonal complex. The initial eBURST diagrams were created at http://eburst.mlst.net and edited as required to produce the final figure. Editing was done to improve the clarity of the diagram and not to change any of the links between the STs and it. The minimum sum of the weights of the edges (genetic distances) was used to create a minimum-spanning tree from MLST profile data. Prim's algorithm was implemented to determine the links between different STs (Prim 1957). The highest priority was given to STs with the most numerous single locus variants. The two STs with the greatest number of single locus and then double locus variants were linked first, preferably using intermediate STs.

2.5 Statistical Analyses

Yates' Chi-square tests were performed on differences of percentage distribution (Ury & Fleiss 1980; Yates 1934). Significance was set at a p value of < 0.05. The Yates' correction prevents overestimation of statistical significance for small data sets. This reduces the chi-square value obtained and thus increases its p-value. Yates' correction is typically used in Chi-square analysis with 1 degree of freedom where expected frequencies of less than 5.

MLST data were analyzed with SAS (SAS Institute Inc., Cary, NC) by using the marascuilo procedure test to determine if there is a significant difference between the ratios for every pair of STs. The trend of change of the prevalence for the gonococcal strains over a four-year period was evaluated using the Chi-square multiple hypothesis test.

Simpson's index of diversity (ID) was used to determine the discriminatory abilities of the molecular typing methods (Hunter & Gaston 1988; Dillon et al 1993). The numerical index is based on the probability that two unrelated isolates would be placed into different typing groups (Hunter & Gaston 1988). The calculation formula is: $ID = 1 - [1/N(N-1)] [\Sigma n_i(n_i-1)]$ where N equals the sum of all of the isolates, and n_i is the size of the i^{th} group. ID is expressed as a percentage. An ID of 90% or greater is desirable for a typing scheme (Hunter & Gaston 1988), depending on the objectives of molecular epidemiological studies.

2.6 DNA Sequence Deposit

DNA sequences were submitted to the National Center for Biotechnology Information (NCBI) GenBank (http://www.ncbi.nlm.nih.gov/WebSub/?tool=genbank). GenBank accession numbers were reported for different housekeeping genes extended from HM359017 to HM359073. The DNA sequences of *porB* and *tbpB* for NG-MAST analysis were stored in the NG-MAST database (http://www.ng-mast.net; Martin et al 2004).

CHAPTER THREE

RESULTS

3.1 Clinical, Demographic and Geographic Profiles of *N. gonorrhoeae* Isolates

N. gonorrhoeae isolates investigated in this research were collected in Saskatchewan (SK) between 2003 and 2011. Of the 427 *N. gonorrhoeae* isolates evaluated, 58.5% (n=250) were isolated from male patients and 177 (41.5%) were from female patients (Fig 3.1). These isolates were 5.1% (356/6965) of the total number of gonorrhea cases reported between 2003 and 2009. The data on number of cases reported in SK is available until 2009 (PHAC 2011a).

Among males, 78.4% (n=196) of the reported isolations were from the urethra, followed by the penis (n=45, 18%) and the throat (n=4, 1.6%). Two gonococcal isolates were recovered from the rectum (0.8%) and one each was isolated from the knee (0.4%) and eye (0.4%). The isolation site for one gonococcal isolate from a male patient was not known. In females, 70% (n=124) of gonococcal isolations were from the cervix followed by 25.4% (n=45) recovered from the vagina. One *N. gonorrhoeae* isolate each was recovered from knee, rectum, throat and bartholin gland in females; 2 gonococcal isolations were made from the peritoneum; and, for two isolates, the sites of isolation were unknown in females (Fig 3.1).

Of the 427 *N. gonorrhoeae* isolates, 422 were collected from 22 known locations across SK (Table 3.1) and for five isolates locations were unknown. Gonococcal cultures were highest from Regina (31%, n=139) followed by Prince Albert (23%, n=99) and Saskatoon (22%, n=96). In both sexes, the highest numbers of gonorrhea cases were recorded for the 20-24 years age group (Fig 3.2). A total of 124 (29%) gonorrhea cases were recorded in this age group comprising 78 (31.2%) male and 46 (26%) female patients. Twenty percent and 17% of cases were grouped under age categories 25-29 and 15-19 years, respectively. The numbers of male gonorrhea patients were higher than female patients in age groups as follows; 20-24 (males: 78/124, 63%; females: 46/124, 37%), 30-34 (males: 30/39, 77%; females: 9/39, 23%), 40-44 (males: 14/19, 74%; females: 5/19, 26%), 45-49 (males: 17/17, 100%; females: 0/17, 0%), 50-54 (males: 8/9, 89%; females: 1/9, 11%), 55-59 (males: 5/5, 100%; females: 0/5, 0%) and above 60 (males: 10/11, 91%; females: 1/11, 9%) years of age. The female patients were more in younger age groups; <1 (females: 1/1, 100%; males: 0/1, 0%), 10-14 (females: 10/10, 100%; males: 0/10, 0%), 15-19 (Females: 47/72, 65.3%; males: 25/72, 34.7%) years of age (Fig 3.2).

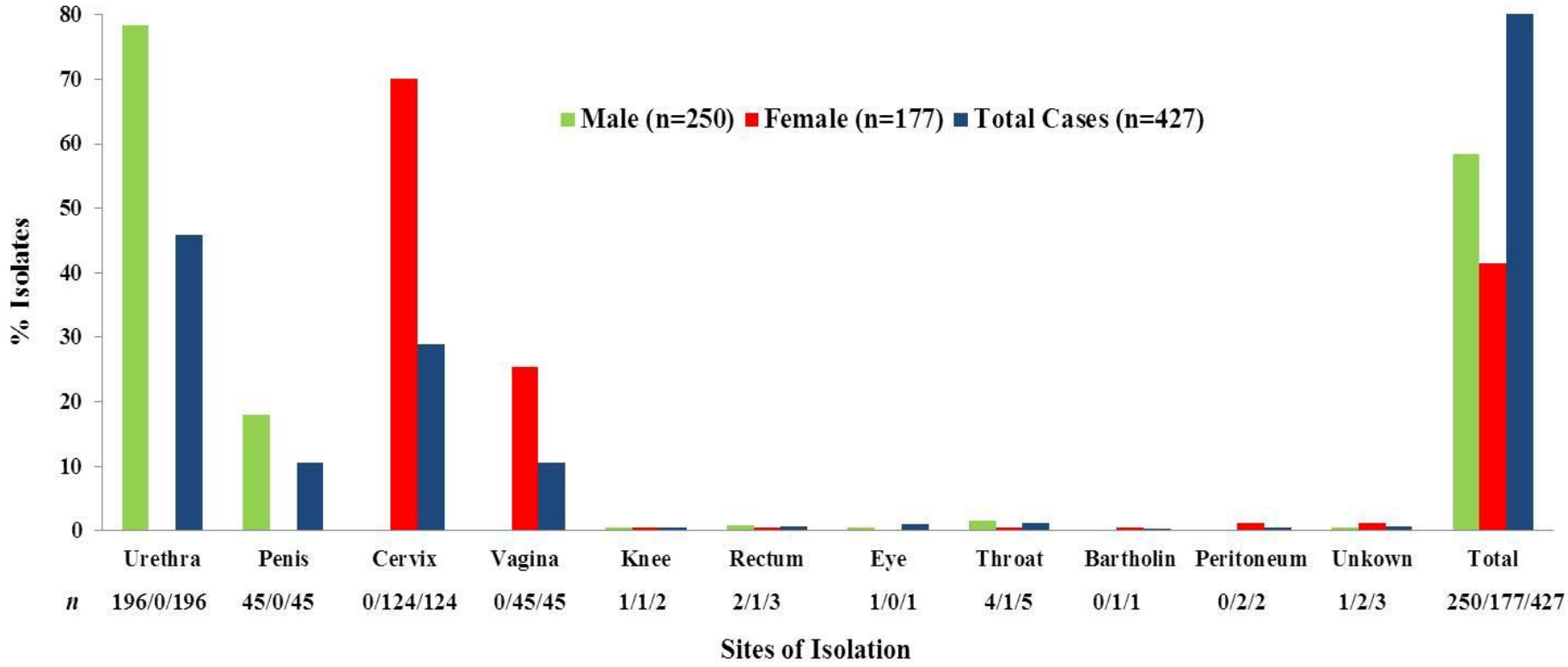

Fig. 3.1 Sites of isolation of 427 *N. gonorrhoeae* isolates from Saskatchewan, Canada: 2003-2011. Lesser number of *N. gonorrhoeae* isolates were recovered from rectum and throat, major infection sites in men having sex with men (MSM), a high risk group for gonorrhea infection.

Table 3.1 Geographic distribution of 427 clinical *N. gonorrhoeae* isolates from Saskatchewan

Geographic Locations	2003 (n=60)		2004 (n=59)		2005 (n=52)		2006 (n=55)		2007 (n=53)		2008 (n=41)		2009 (n=36)		2010 (n=34)		2011 (n=37)		Total (n=427)		Grand Total (n=427)
	M	F	M	F	M	F	M	F	M	F	M	F	M	F	M	F	M	F	M	F	M + F
Ile a la Crosse	0	0	0	4	0	0	0	1	0	1	0	2	0	1	1	0	0	0	1	9	10
La Ronge	1	3	0	4	1	1	0	3	3	1	1	3	0	1	0	0	0	0	6	16	22
Nipawin	0	3	0	1	0	0	0	0	0	0	0	1	0	4	0	1	0	0	0	10	10
Prince Albert	12	6	9	2	1	3	11	7	12	1	9	2	7	4	5	2	4	2	70	29	99
Regina	15	6	15	12	10	7	6	4	3	3	4	5	7	3	18	3	8	10	86	53	139
Saskatoon	7	4	4	4	12	9	12	5	11	5	5	3	5	1	0	0	9	2	65	33	98
Yorkton	0	0	0	2	1	0	0	0	3	1	0	2	1	0	0	1	0	0	5	6	11
Other locations[a]	1	2	0	2	5	2	3	3	2	7	2	2	1	1	1	2	2	0	17	21	38
Total	36	24	28	31	30	22	32	23	34	19	21	20	21	15	25	9	23	14	250	177	427

a: 38 isolates were recovered from 24 different locations across the province.
M: male
F: female

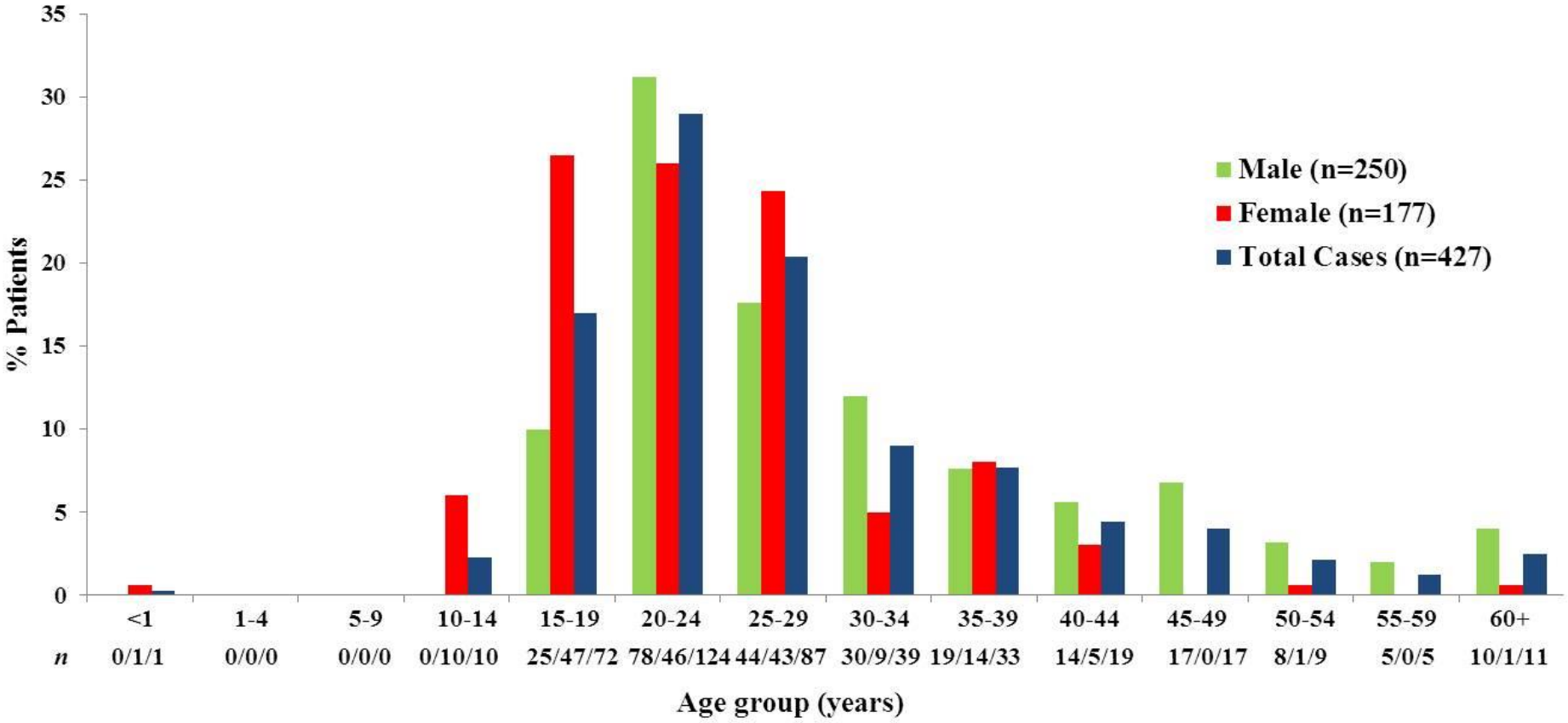

Fig. 3.2 Age and sex wise distribution of gonorrhea patients in Saskatchewan, Canada: 2003-2011. Highest number of cases were reported from age group between 20-24 years, followed by age groups between 25-29 and 15-19.

The number gonococcal cultures isolated from gonorrhea patients in the province is decreasing (Fig 3.3). In 2003, 11% (n=60) of 544 reported gonorrhea cases were cultured and this decreased to 3.2% (n=41) of reported cases (n=1334) in 2008. In 2009, 36 (4.1%) gonococcal isolates were cultured and reported cases of gonorrhea in SK for the year 2009 were 875.

3.2 Temporal and Geographic Trends of Antimicrobial Resistance in *Neisseria gonorrhoeae* in Saskatchewan (2003-2011)

(Please see tables A.1 to A.7 from page 241 to 247 of appendix-I. These tables show raw data on penicillin, tetracycline, ciprofloxacin, azithromycin, spectinomycin, cefixime and ceftriaxone susceptibility of *N. gonorrhoeae* isolates from SK between 2003 and 2011).

The antimicrobial susceptibility of 427 clinical *N. gonorrhoeae* isolates consecutively collected from SK (2003-2011) was examined against penicillin, tetracycline, ciprofloxacin, azithromycin, spectinomycin, cefixime and ceftriaxone (Table 3.2). Penicillin MICs ranged between 0.001 and 32 mg/L, with 4.0% (n=17) of the tested isolates being resistant (MIC≥ 2 mg/L) to penicillin. Penicillin had $MICs_{50}$ (n=271) and $MICs_{90}$ (n=410) values of 0.25 mg/L and 1.0 mg/L.

Tetracycline MICs ranged from 0.008 to 256 mg/L and the total burden of tetracycline resistance during 2003-2011 was 50% (n=214/427) (Table 3.2). $MICs_{50}$ (n=211) and $MICs_{90}$ (n=413) values for tetracycline were 1.0 mg/L and 8.0 mg/L.

Ciprofloxacin MICs ranged from 0.001 mg/L to 32 mg/L and 19 (4.4%) *N. gonorrhoeae* isolates were resistant to ciprofloxacin (Table 3.2). Isolates had ciprofloxacin $MICs_{50}$ (n=256) and $MICs_{90}$ (n=405) values of 0.004 mg/L and 0.016 mg/L.

Azithromycin resistance was found in 0.54% (n=2) of 320 isolates tested (2003-2008). MIC values for azithromycin ranged between 0.016 to 8.0 mg/L (Table 3.2). The MIC_{50} (n=225)

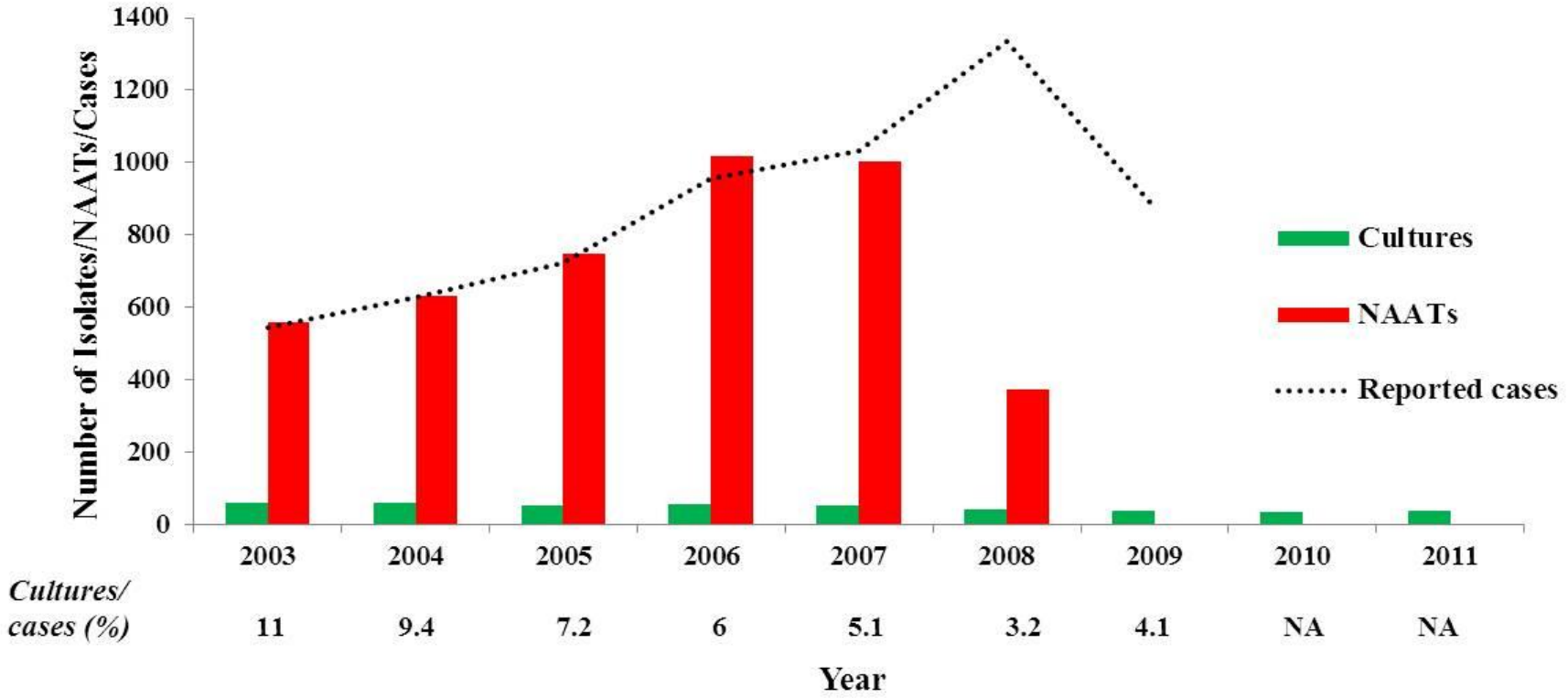

Fig. 3.3 Number of gonorrhea cases diagnosed with cultures and nucleic acid amplification tests (NAATs) in Saskatchewan, Canada: 2003-2011. Data on reported cases was derived from Public Health Agency of Canada (PHAC 2011a). The number of NAATs done to diagnose gonorrhea cases in SK were provided by SDCL, Regina, SK. Data on reported cases of was not available for the years 2010 and 2011. NAATs data was available till 2008.

Table 3.2 Antibiotic susceptibility of 427 *N. gonorrhoeae* isolates from Saskatchewan (2003-2011)[a]

Antibiotic	% Susceptible (n)	%Intermediate (n)	% Resistant[b] (n)	MIC range (mg/L)	MIC_{50} (mg/L)	MIC_{90} (mg/L)
Penicillin	25.0 (105)	71.0 (305)	4.0 (17)	0.001-32.0	0.25	1.0
Tetracycline	29.9 (128)	20.0 (85)	50.1 (214)	0.008-256.0	1.0	8.0
Ciprofloxacin	95.0 (406)	0.5 (2)	4.5 (19)	0.001-32.0	0.004	0.016
Azithromycin[c]	99.5 (318)	0 (0)	0.5 (2)	0.016-8.0	0.25	0.5
Spectinomycin	96.7 (413)	3.3 (14)	0 (0)	4.0-64.0	16.0	32.0
Cefixime	100 (427)	ND[d]	ND	0.0005-0.125	0.008	0.016
Ceftriaxone	100 (427)	ND	ND	0.00025-0.025	0.008	0.03

a: Interpretative criteria for MIC classification were those described by the CLSI (CLSI 2009). Azithromycin MIC classification criteria are not described by CLSI, therefore standards followed by Gonococcal Isolate Surveillance Project (GISP), USA were used (CDC 2010b).

b: Break points are penicillin$\geqslant$2 mg/L; tetracycline$\geqslant$2 mg/L; ciprofloxacin$\geqslant$1 mg/L; azithromycin$\geqslant$2 mg/L; spectinomycin $\geqslant$ 128 mg/L. Break points are not available for cefixime and ceftriaxone and MICs below 0.25 mg/L are considered susceptible.

c: *N. gonorrhoeae* isolates from 2003 to 2008 (n=320) were tested for azithromycin susceptibility.

d: Break points are not defined.

and MIC_{90} (n=304) values of *N. gonorrhoeae* isolates to azithromycin were 0.25 mg/L and 0.5 mg/L.

All isolates were susceptible to spectinomycin (MIC<128 mg/L; MIC range 4.0–64.0 mg/L) (Table 3.2). 3.3% (n=14) of the tested gonococcal isolates had intermediate (MIC=64 mg/L) levels of resistance. MIC_{50} (n=344) and MIC_{90} (n=413) values for spectinomycin were 16.0 mg/L and 32.0 mg/L.

All isolates were susceptible to cefixime (MIC range 0.0005-0.125 mg/L) (Table 3.2). One isolate with a cefixime MIC of 0.125 mg/L was isolated in 2006. The MIC_{50} (n=281) and MIC_{90} (n=399) values for cefixime were 0.008 mg/L and 0.016 mg/L. Ceftriaxone MICs ranged between 0.00025 and 0.25 mg/L (Table 3.2). Ceftriaxone had MIC_{50} and MIC_{90} values of 0.008 mg/L (n=286) and 0.03 mg/L (n=410).

No penicillin resistant isolate was detected in 2006, 2007 and 2010 (Fig 3.4). Penicillin resistance ranged from 2.7% in 2009 (1/36) and 2011 (1/37) to 6.8% in 2003 (4/60) and 2004 (4/59). Penicillin resistance in the province remained below 5% from 2006 to 2011. One PPNG isolate was detected in each of 2003, 2005, 2009 and 2011.

The prevalence of CMTR (n=200/427) was 47%. TRNG (Tet MIC ≥16 mg/L; n=14/427) constituted 3.3% of the total tetracycline resistance (Fig 3.5). CMTR contribution to tetracycline resistance was 76.3% (42/55) in 2006 followed by 85% (45/53) in 2007. In 2008, 73% (30/41) of the isolates recovered that year were resistant to tetracycline. This declined to 0% in 2009 and 2010.

Ciprofloxacin resistant isolates were not observed between 2003 and 2005 and in 2009 (Fig 3.6). Ciprofloxacin resistance in the province remained below 5% till 2009. In 2010 and 2011, 26.5% (9/35) and 13.5% (5/37) isolates were resistant to ciprofloxacin.

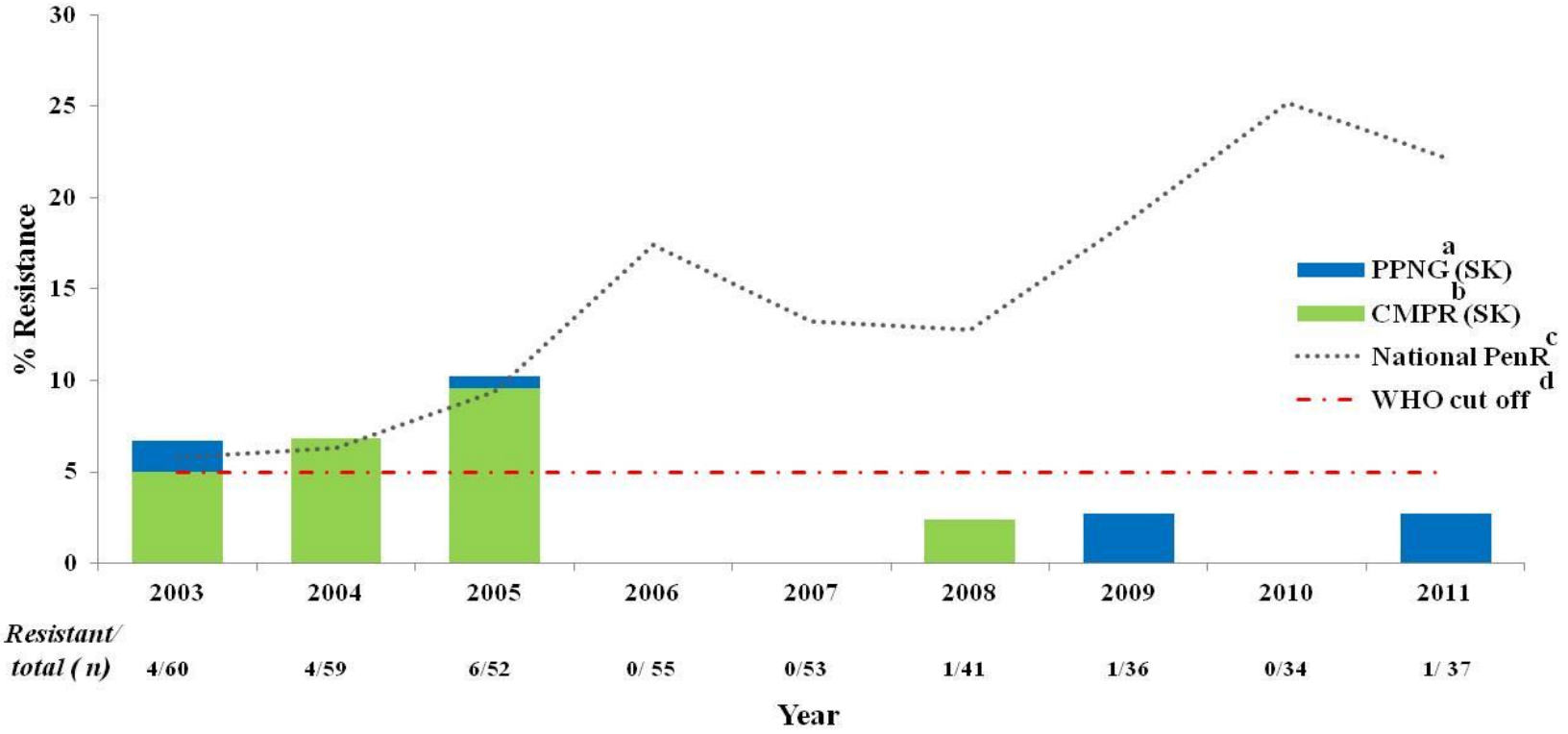

Fig. 3.4 Percent penicillin resistant *N. gonorrhoeae* isolates from Saskatchewan: 2003-2011.

[a]Penicillinase producing *N. gonorrhoeae*; [b]chromosomally mediated Pen resistance; [c]national Canadian Pen resistance rates for *N. gonorrhoeae*; [d]WHO recommends change in treatment guidelines at the levels of 5% antibiotic resistance in vitro.

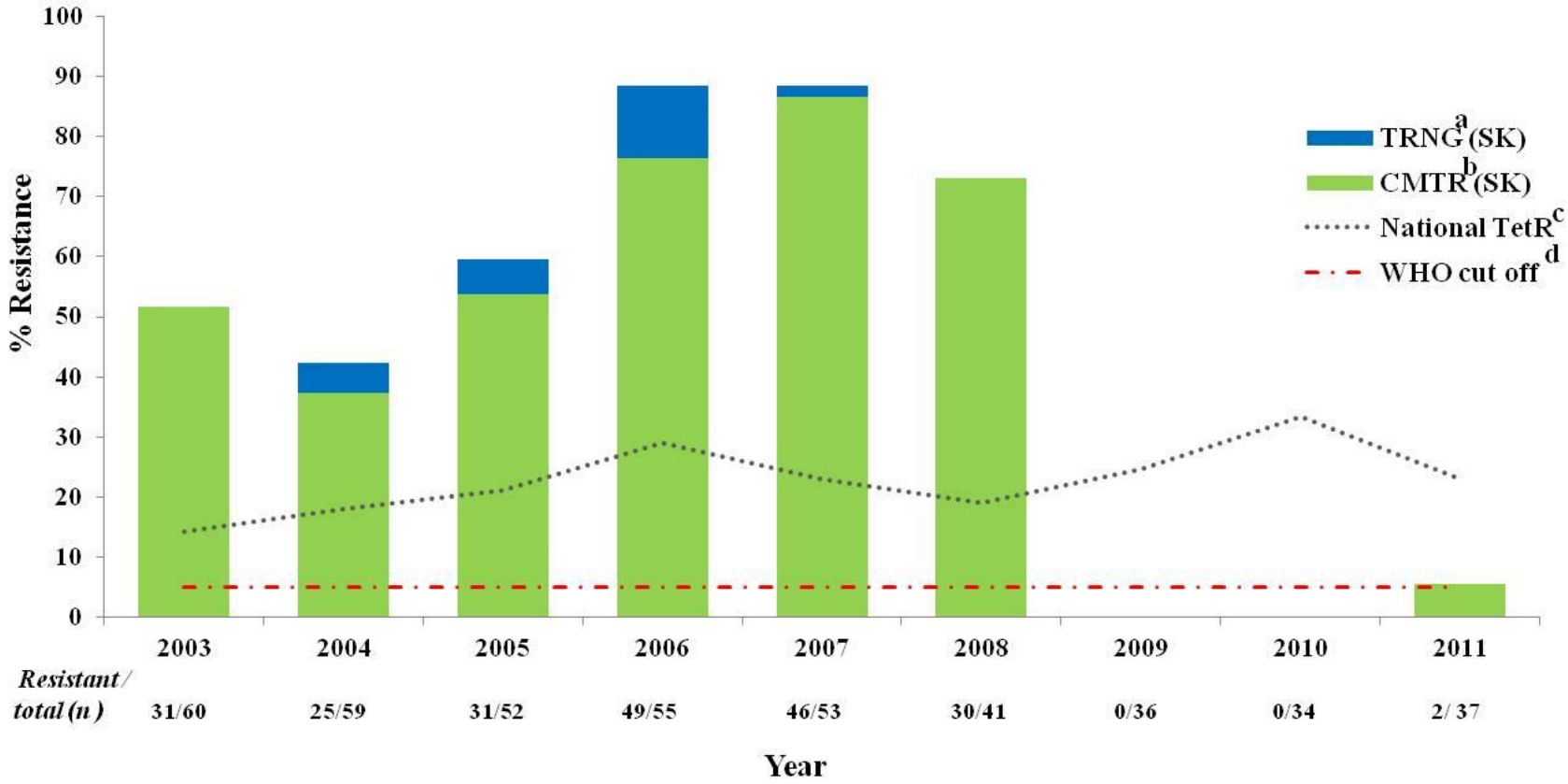

Fig. 3.5 Percent tetracycline resistant *N. gonorrhoeae* isolates from Saskatchewan: 2003-2011.

[a]Tet resistant *N. gonorrhoeae*; [b]chromosomally mediated Tet resistance; [c]national Canadian Tet resistance rates for *N. gonorrhoeae*; [d]WHO recommends change in treatment guidelines at the levels of 5% antibiotic resistance in vitro.

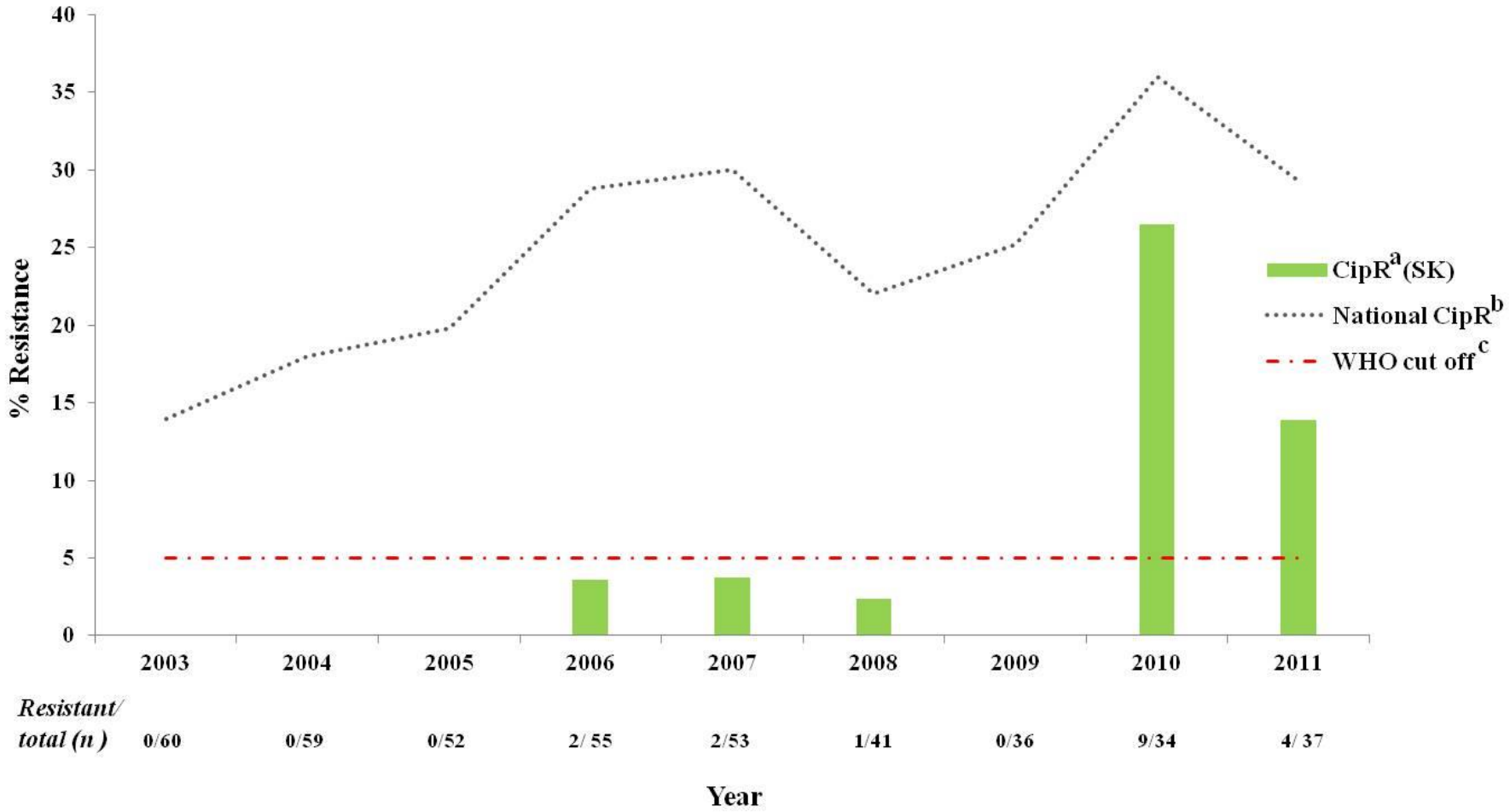

Fig. 3.6 Percent ciprofloxacin resistant *N. gonorrhoeae* isolates from Saskatchewan: 2003-2011.

[a]Cip resistant *N. gonorrhoeae*; [b]national Canadian Cip resistance rates for *N. gonorrhoeae;* [c]WHO recommends change in treatment guidelines at the levels of 5% antibiotic resistance in vitro.

Azithromycin resistant isolates were recovered in 2004 (1.7%, 1/59) and 2007 (1.9%, 1/53) (Fig 3.7).

No cefixime MIC creep was observed and no clear trend was detected for cefixime susceptibility (Fig. 3.8). More than 52% (n=220) of the *N. gonorrhoeae* isolates had cefixime MICs lower than 0.008 mg/L with a further 118 (28%) isolates having MICs of 0.016 mg/L.

One isolate each with ceftriaxone MIC between 0.25 mg/L and 0.125 mg/L was recovered in 2010 and 2011 (Fig. 3.9). Around 24% (104/427) of the isolates each had ceftriaxone MICs between 0.004 mg/L and 0.008 mg/L and other 19% (83/427) were with ceftriaxone MIC 0.016 mg/L.

Overall 10 resistance phenotypes were found in 427 isolates from SK. The resistance phenotypes included: susceptible (n=194, 45%), CMTR (n=183, 43%), ciprofloxacin resistance (Cip^R, n=16, 3.75), CMRNG (n=13, 3%), TRNG (n=13, 3%), PPNG (n=3, 0.7%), CMTR; Cip^R (n=2, 0.4%), PPNG (n=1, 0.2%), TRNG; Cip^R (n=1, 0.2%), azithromycin resistance (Azi^R, n=1, 0.2%).

The majority of *N. gonorrhoeae* isolates resistant to different antibiotics detected in this study were from 3 cities of SK; Saskatoon, Regina and Prince Albert (Fig 3.10). Seventy-nine percent (195/247) of the total resistant isolates were from Saskatoon, Regina and Prince Albert. A higher percentage of total penicillin (71%, 12/17; P=0.0016), ciprofloxacin (53%, 10/19; P=0.096) and tetracycline (29%, 62/214; P=0.14) resistant *N. gonorrhoeae* isolates were from Regina compared to the other locations. One of the two azithromycin resistant isolates was also recovered from Regina. Isolates from Saskatoon contributed 24% (4/17; P=0.095), 32% (6/19; P=0.52) and 26% (55/214; P=0.2) to penicillin, ciprofloxacin and tetracycline resistance, respectively in SK. No penicillin resistant gonococcal isolate was detected in Prince Albert. Eleven percent (2/19; P=0.29) and 20% (42/214; P=0.1027) of total ciprofloxacin and tetracycline resistant *N. gonorrhoeae* isolates in SK detected during 2005-2008 were from Prince Albert. One PPNG and ciprofloxacin resistant isolate was recovered from Yorkton and Lloydminster.

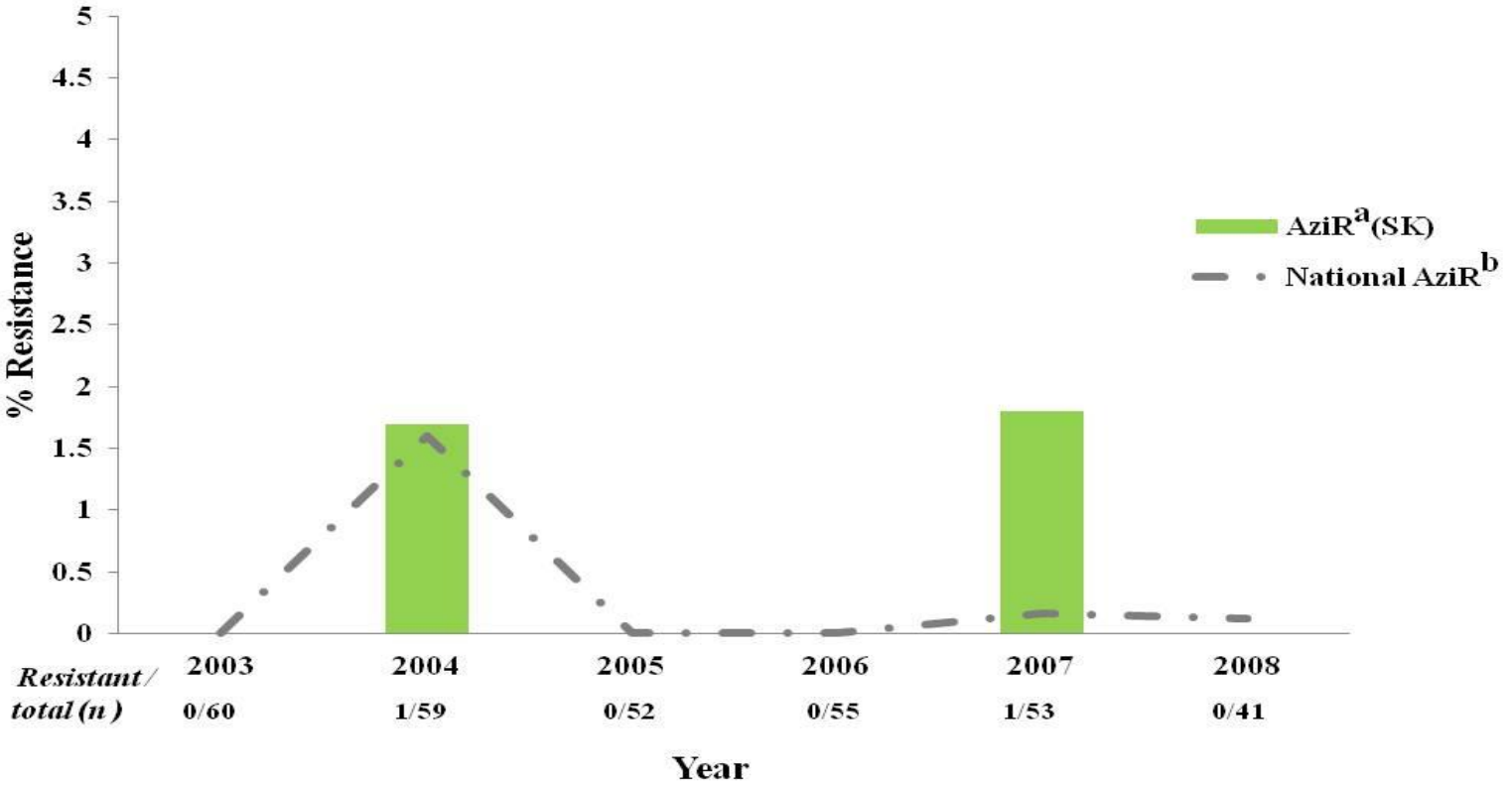

Fig. 3.7 Percent azithromycin resistant *N. gonorrhoeae* isolates from Saskatchewan: 2003-2008.

[a]Azi resistant *N. gonorrhoeae*; [b]WHO recommends change in treatment guidelines at the levels of 5% antibiotic resistance in vitro.
National Canadian Azi resistance rates are not available.

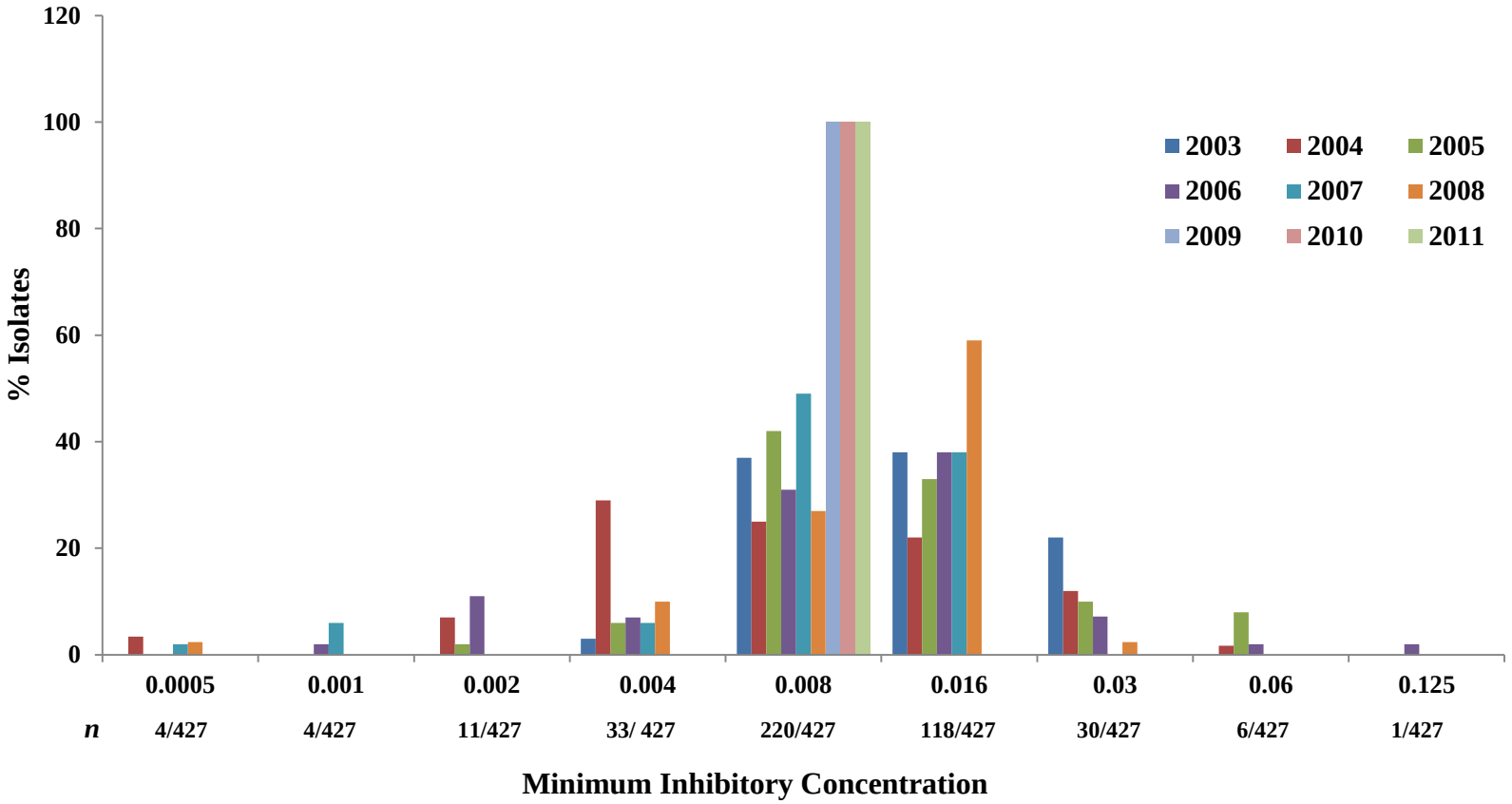

Fig. 3.8 Cefixime minimum inhibitory concentration wise distribution *N. gonorrhoeae* isolates from Saskatchewan: 2003-2011. 220 (52%) *N. gonorrhoeae* isolates each were grouped under cefixime MIC 0.008 mg/L followed by 118 (28%) under MIC 0.016. All the isolates from 2009, 2010 and 2011 had cefixime MIC 0.008 mg/L .

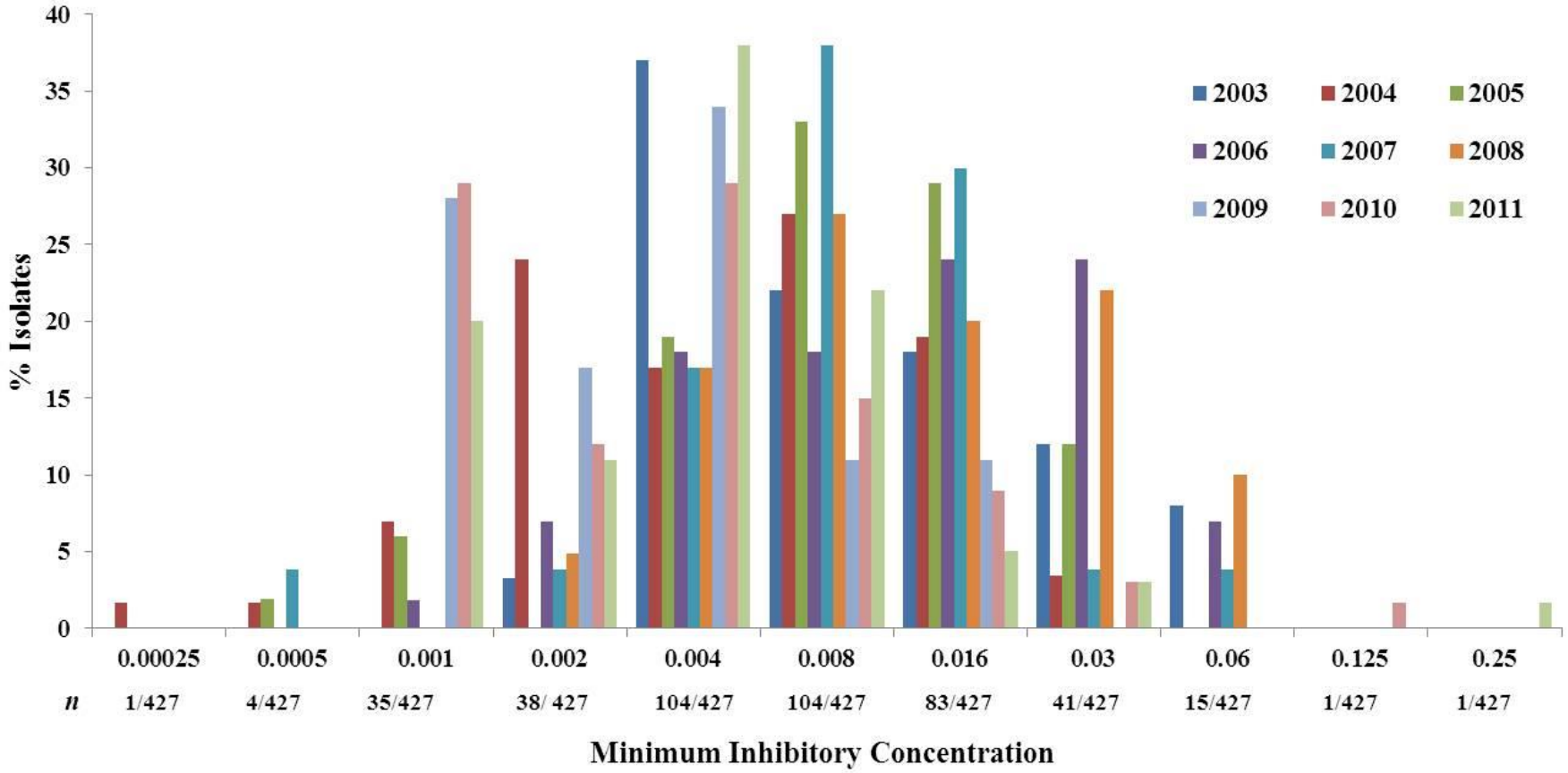

Fig. 3.9 Ceftriaxone minimum inhibitory concentration wise distribution *N. gonorrhoeae* isolates from Saskatchewan: 2003-2011. 104 (24%) *N. gonorrhoeae* isolates each were grouped under ceftriaxone MIC 0.004 mg/L and 0.008 mg/L followed by 83 (19%) under MIC 0.016.

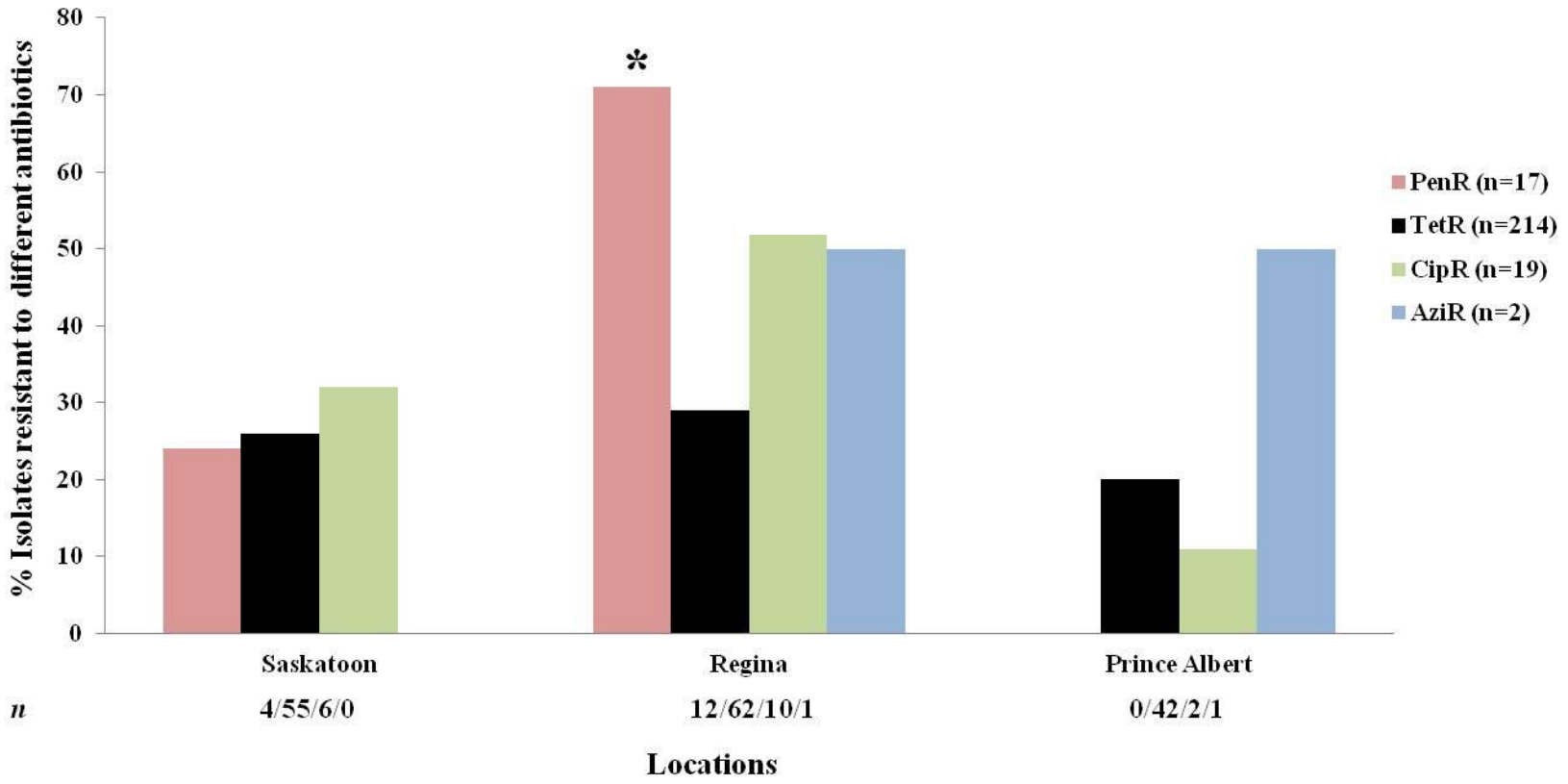

Fig. 3.10 Distribution of antibiotic resistant *N. gonorrhoeae* isolates from Saskatchewan: 2003-2011

*: P value < 0.05 was considered statistically significant.

3.3 DNA Sequence Analysis of Genes implicated in Antomicrobial Resistance in *Neisseria gonorrhoeae*

3.3.1 DNA Sequence Analysis of *penA*, *porB*, *ponA* and *mtrR* implicated in Reduced Susceptibility to Cephalosporins

DNA sequences of *penA* (PBP2), *porB* (PorB) *ponA* (PBP1) and *mtrR* (MtrR and its promoter) were analyzed in 146 *N. gonorrhoeae* isolates to determine emerging molecular mechanisms of extended spectrum cephalosporin susceptibility. Isolates were divided into two groups: isolates with MICs 0.0005-0.016 mg/L (cefixime group 1; n=122 & ceftriaxone group 1; n=123) and isolates with MICs 0.03-0.06 mg/L (cefixime group 2; n=24 & ceftriaxone group 2; n=23).

i) *penA* (PBP2) Analysis

(Please see table A.8 in appendix-I showing raw data on PBP2 mutations in *N. gonorrhoeae* isolates analyzed for resistance determinants of extended spectrum cephalosporins).

Analysis of the transpeptidase domain (amino acids 340 to 575) (Dougherty et al 1980) of PBP2 (581 amino acids) revealed nine patterns in 146 *N. gonorrhoeae* isolates. Seven of which were non-mosaic (spontaneous mutations) *penA* patterns (I, II, V, IX, XII, XIV, XXII). One PBP2 pattern, XXXIV, was mosaic with more than 30 amino acid substitutions (Table 3.3). This mosaic pattern has been reported to arise due to homologous recombination between *N. gonorrhoeae penA* and *penA* of commensal *Neisseria* (Pandori et al 2009). The majority of isolates investigated (86%, 126/146) were associated with patterns IX (n=50, 34%), I (n=41, 28%), and XXII (n=35, 24%).

All non-mosaic PBP2 patterns detected in this study had an Asp-346 insertion (Fig 3.11). Amino acid substitutions (F504, A510 & A516) between AA340 and AA540 of PBP2 were identical in *penA* pattern II, V, IX, XII, XIV and XXII. Pattern IX was significantly associated with ceftriaxone group 2 isolates (MIC range 0.03-0.06 mg/L; 14/23, 61%; P=0.007) (Fig 3.12).

Table 3.3 Mutation patterns of gonococcal PBP2 in isolates from Saskatchewan

PBP2 patterns[a]	n[b](%)	Amino acid substitutions at the transpeptidase domain
I	41 (28.0)	D345a
II [c]	7 (5.0)	D345a, F504L, A510V, A516G
IX	50 (34)	D345a, F504L, A510V, A516G, P551L
XII	6 (4.0)	D345a, F504L, A510V, A516G, P551S
XIV	2 (1.5)	D345a, F504L, A510V, A516G, H541N
V	2 (1.5)	D345a, F504L, A510V, A516G, G542S, I566V, N574a, A575V
XXII	35 (24)	D345a, F504L, A510V, A516G, H541N, P552V, K555Q, I556V, I566V, N574a, A575V
XXXIV[d]	1 (0.7)	P341S, S342A, P343T, R345Q, S352T, R373M, G375T,A376P, E377K, E385D,I388V, N406S, R411Q, P412K, A437V, V443E, L447V, Q457K, I461V, F462I, E464A, R468K, E469K, N472E, P480A, F504L, A510V, N512Y, H541N, G545S

a: Mutation patterns were determined based on amino acid substitutions in the regions of AA340 to AA575 of *N. gonorrhoeae* PBP2. Two isolates were with WT PBP2.
b: Two isolates were with WT transpeptidase domain.
c: Pattern I, II, V, IX, XII, XIV, XXII had identical amino acid substitution in the region of AA340-AA540 of PBP2 which were compared to WT (M32091; Spratt 1998) transpeptidase domain.
d: Pattern XXXIV mosaic PBP2.

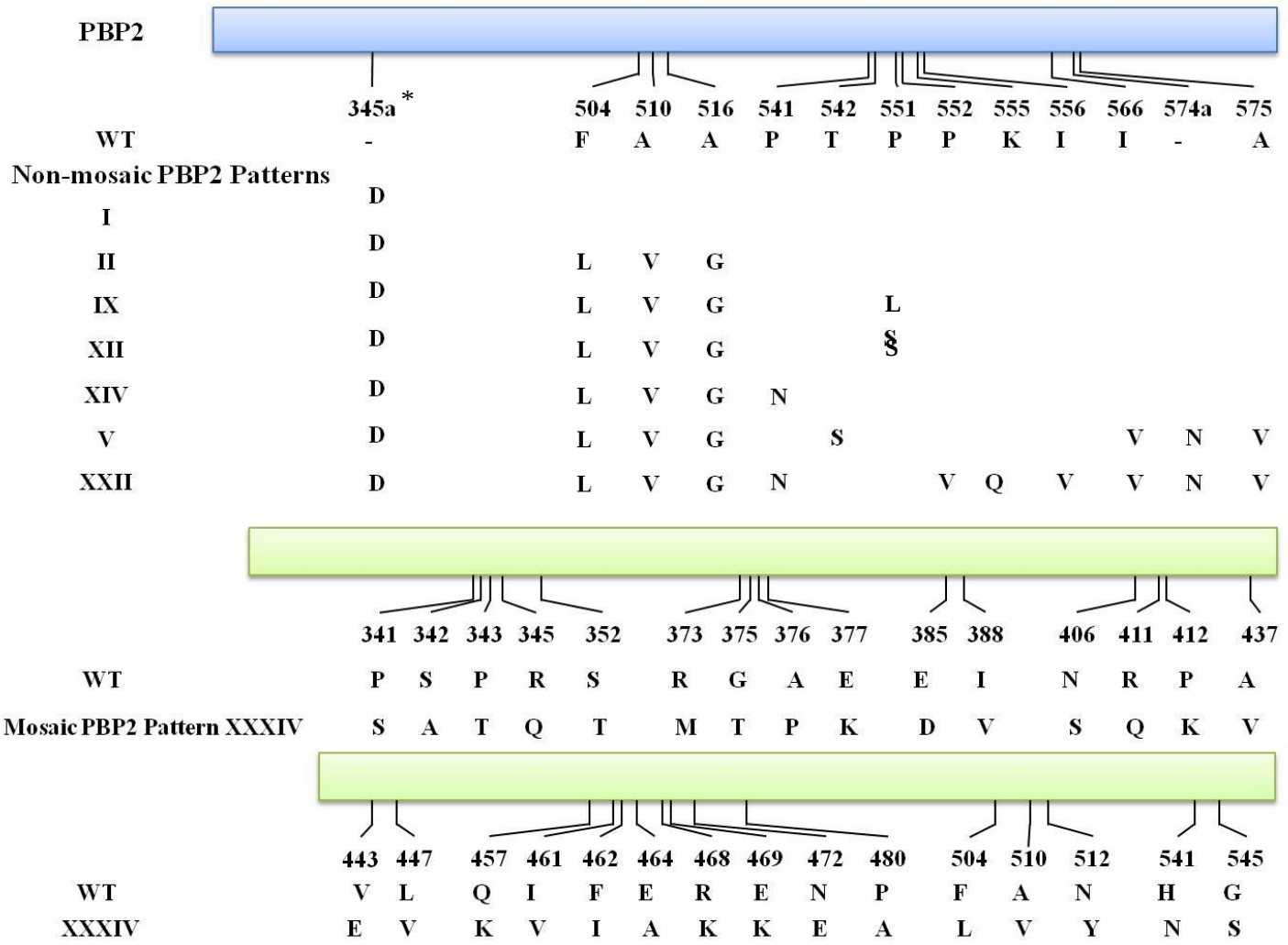

Fig. 3.11 PBP2 mutation patterns in 146 *N. gonorrhoeae* isolates from Saskatchewan: 2003-2008. *N. gonorrhoeae* PBP2 has 581 amino acids. Mutations were determined based on amino acid substitutions in the transpeptidase domain of *N. gonorrhoeae* PBP2 between AA340 and AA575 (Dougherty et al 1980; Ito et al 2005; Whiley et al 2007a; Ohinishi et al 2011b). Pattern II, V, IX, XII, XIV, XXII were identical from AA340-[illegible] attern XXXIV is a mosaic PBP2 pattern.
WT: Wild type PBP2.
*: 345a- insertion after AA345.

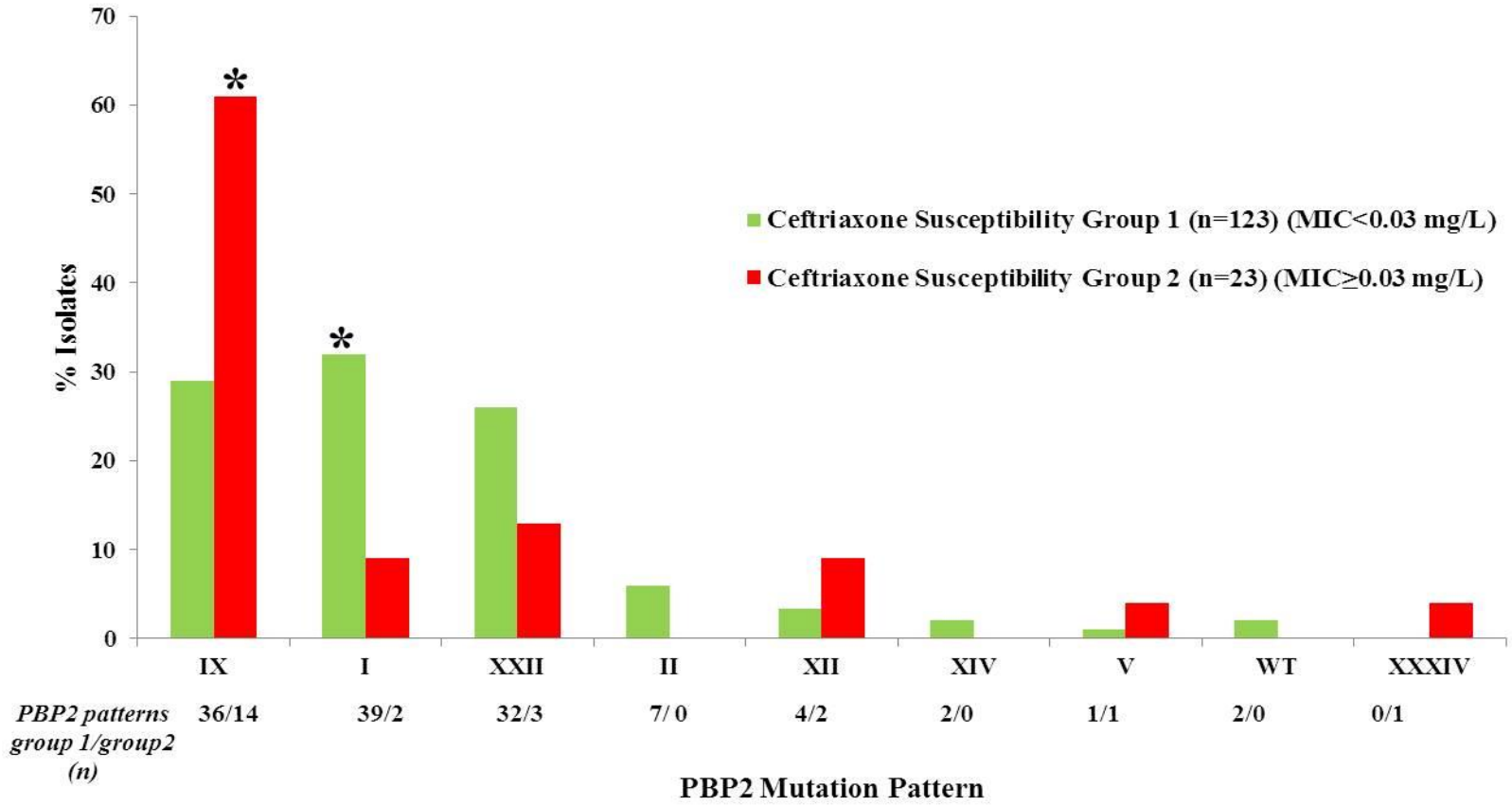

Fig. 3.12 PBP2 mutation patterns in 146 *N. gonorrhoeae* isolates analyzed for molecular determinants of ceftriaxone susceptibility. Amino acid substitutions between AA340 to AA575 were determined. Percentages of PBP2 mutation patterns were presented for ceftriaxone susceptibility group 1 (MICs=0.0005-0.03 mg/L) and ceftriaxone susceptibility group 2 (MICs=0.03-0.06 mg/L) isolates.
*: P value < 0.05 was considered statistically significant.

Pattern I was significantly associated to ceftriaxone group 1 isolates (MIC range 0.0005-0.016 mg/L; 39/123, 32%; P=0.045). Pattern XXII was mostly found in ceftriaxone group 1 isolates (32/123, 26%). Patterns less common in this study i.e. II, XII, XIV, V and XXXIV (in total, n=20) were mostly found in ceftriaxone group 1 isolates (16/20). *N. gonorrhoeae* isolate with mosaic PBP2 pattern XXXIV (ceftriaxone MIC=0.06 mg/L; cefixime MIC=0.125 mg/L), was identified in ceftriaxone group 1 (Fig 3.12).

Similar distribution patterns for PBP2 were observed with cefixime susceptibility. Pattern IX was significantly associated with cefixime susceptibility group 2 (MIC range 0.03-0.125 mg/L; 16/23, 67%; P=0.006) and pattern I was associated with cefixime susceptibility group 1 (MIC range 0.00025-0.016 mg/L; 39/122, 32%; P=0.045) isolates. There was no significant association in between cefixime MIC and any other PBP2 pattern and most isolates were from cefixime susceptibility group 1.

ii) *porB* (PorB) Analysis

(Please see table A.9 in appendix-I showing raw data on PorB mutations in *N. gonorrhoeae* isolates analyzed for resistance determinants of extended spectrum cephalosporins).

Twelve mutation patterns were observed in porin protein, PorB (Fig 3.13). Analysis of PorB showed that most (53%, 78/146) isolates carried double mutations at amino acid residues G120 and A121. Combined mutations at residues G120 and A121 with charged amino acids [Lys (K) at residue G120 and Asp (D) at residue A121] were significantly associated with *N. gonorrhoeae* ceftriaxone susceptibility group 2 (9/23, 39%; P=0.003) and cefixime susceptibility group 2 (9/24, 38%; P=0.001). Wild type (WT) PorB was identified in 63 isolates mostly from ceftriaxone (60/123, 49%; P=0.003) and cefixime (59/123, 48%; P=0.008) susceptibility groups 1. The distribution of the PorB mutations was the same in ceftriaxone and cefixime susceptibility groups 1 and 2.

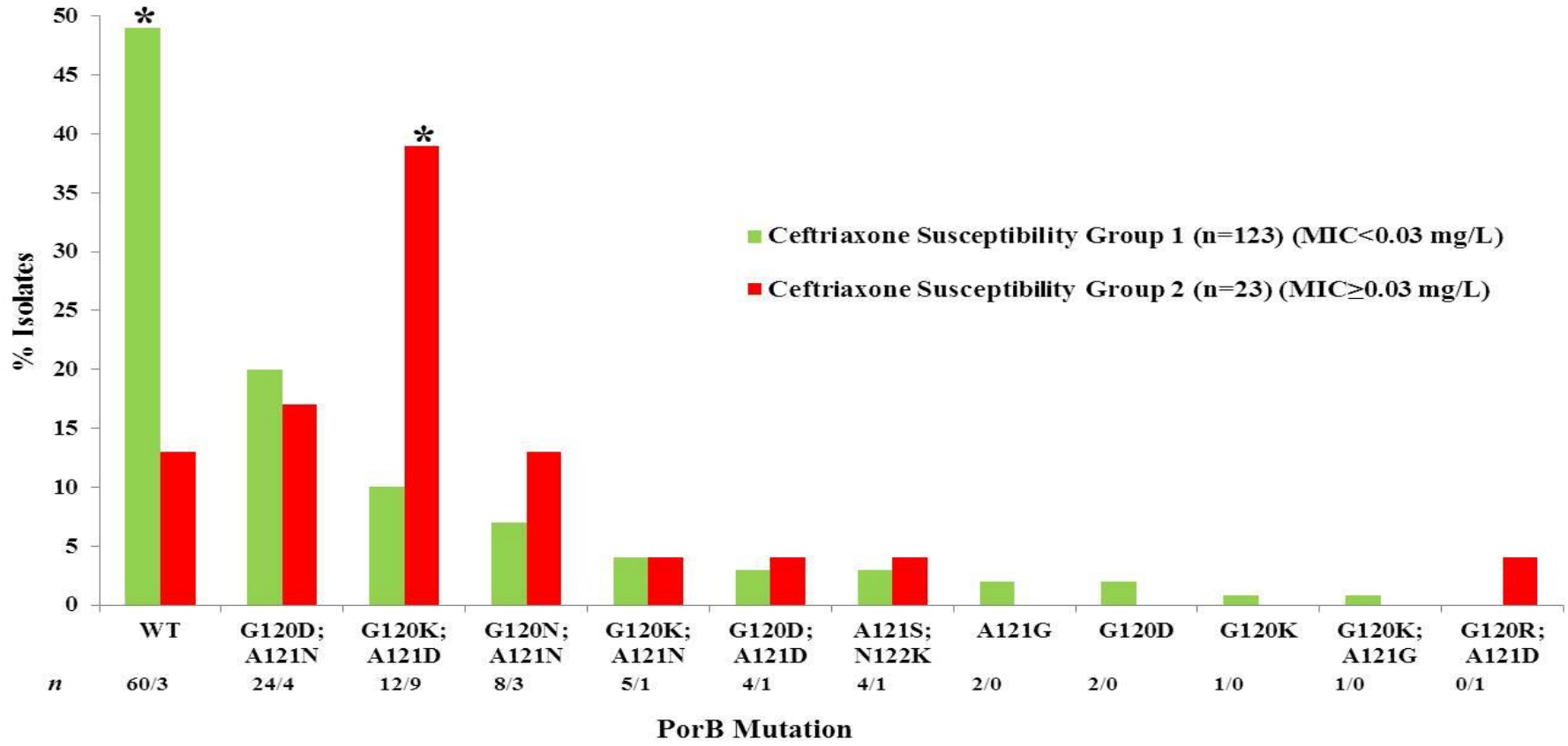

Fig. 3.13 PorB mutation at G120 and A121 in 146 *N. gonorrhoeae* isolates analyzed for molecular determinants of ceftriaxone susceptibility. Amino acid substitutions at G120 and A121 were determined. Percentages of PorB mutations were presented for ceftriaxone group 1 and ceftriaxone group 2 isolates.
*: P value < 0.05 was considered statistically significant.

iii) *ponA* (PBP1) Analysis

More than 60 % of the 146 *N. gonorrhoeae* isolates investigated (89/146, 61%) carried a L421P amino acid substitution in PBP1. This substitution was significantly associated with ceftriaxone and cefixime group 2 isolates (20/23, 87%; P=0.01, data not shown).

iv) *mtrR* (MtrR and its promoter) Analysis

(Please see table A.10 in appendix-I showing raw data on *mtrR* mutations in *N. gonorrhoeae* isolates analyzed for resistance determinants of extended spectrum cephalosporins).

The analysis of *mtrR* and its promoter revealed 12 different mutation patterns derived from mutations in different regions of MtrR (Fig 3.14). The frequencies of different mutation patterns in *mtrR* and its promoter were the same in ceftriaxone and cefixime susceptibility groups. Overall, among the 146 isolates tested, a G45D substitution in the MtrR DNA binding domain predominated (38%, 55/146), both in ceftriaxone susceptibility group 2 (13/23, 57%, P=0.072) and cefixime susceptibility group 2 (12/24, 50%, P=0.25).

The substitution G45D coupled with an adenine deletion (A-) in the 13bp inverted repeat region of the promoter (A-/G45D) was present in 17% (25/146) of the analyzed isolates. The double mutation, A- coupled with H105Y substitution in the multimeric region of MtrR was also significantly associated with both ceftriaxone susceptibility group 2 (5/23, 22%; P=0.008) and cefixime susceptibility group 2 (5/24, 21%, P=0.016) isolates. Wild type *mtrR* sequences were associated with ceftriaxone (37/123, 30%, P=0.005) and cefixime (36/123, 30%, P=0.018) group 1 isolates.

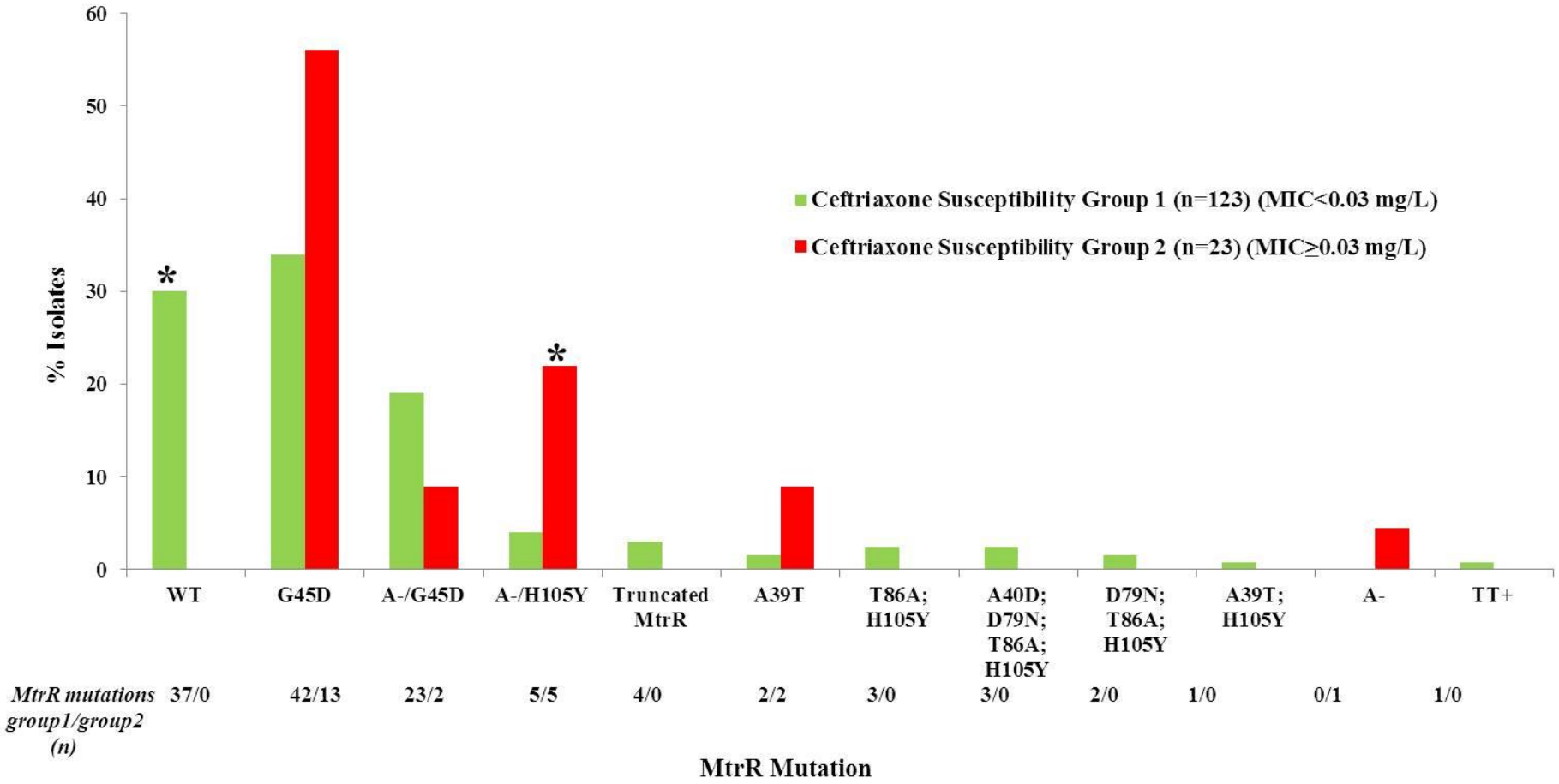

Fig. 3.14 Mutations in MtrR and its promoter in 146 *N. gonorrhoeae* isolates analyzed for ceftriaxone susceptibility. Amino acid substitutions in MtrR and its promoter were determined. Percentages of MtrR mutations were presented for ceftriaxone susceptibility group 1 and ceftriaxone group susceptibility 2 isolates.
*: P value < 0.05 was considered statistically significant.

3.3.2 Analysis of Genes implicated in Azithromycin Resistance

Fifty-two isolates were investigated for azithromycin resistance which included all resistant (MIC≥2.0 mg/L) isolates (n=2), 11 intermediately susceptible (MIC=1.0 mg/L) and 39 susceptible (MIC= 0.03-0.5 mg/L) isolates (Fig 3.15). Both azithromycin resistant *N. gonorrhoeae* isolates had the C2611T mutation in all the four alleles of 23S rRNA and MtrR mutation G45D (P=0.001). Among intermediately susceptible (n=10/11, 91%) and susceptible (9/39, 23%) isolates only the MtrR mutation was detected. In *N. gonorrhoeae* isolates with intermediate susceptibility to azithomycin, *mtrR* mutations were A39T (n=1/11, 9%), A-/H105Y (n=1/11, 9%), G45D (n=3/11, 27%) and A-/G45D (5/11, 45%; P=0.0008). None of these 52 isolates were with rRNA methylase genes (*erm*).

3.3.3 Analysis of Quinolone Resistance determining Region

In total, 41 isolates were investigated for ciprofloxacin resistance mechanisms that included all 5 resistant (ciprofloxacin MIC≥1.0 mg/L) isolates recovered, 2 with intermediate susceptibility (MIC=0.5 mg/L) and 18 susceptible (MIC= 0.002-0.008 mg/L) isolates (Table 3.4).

Ciprofloxacin resistant isolates carried GyrA-S91F/D95G; ParC-S87R (n=4/5, 80%; P=0.0005) and GyrA-S91F/D95N; ParC-S87R (n=1) mutations. The intermediately ciprofloxacin susceptible isolates (n=2) carried mutations; GyrA-S91F/D95G; ParC-S87R and GyrA-S91F/D95N; ParC-S87R/S88P. Ciprofloxacin resistance was also significantly associated with *mtrR* mutation A-/H105Y (4/5, 80%; P=0.01) and ciprofloxacin intermediate susceptibility (2/2, 100%) was significantly linked with truncated *mtrR* (P=0.01).

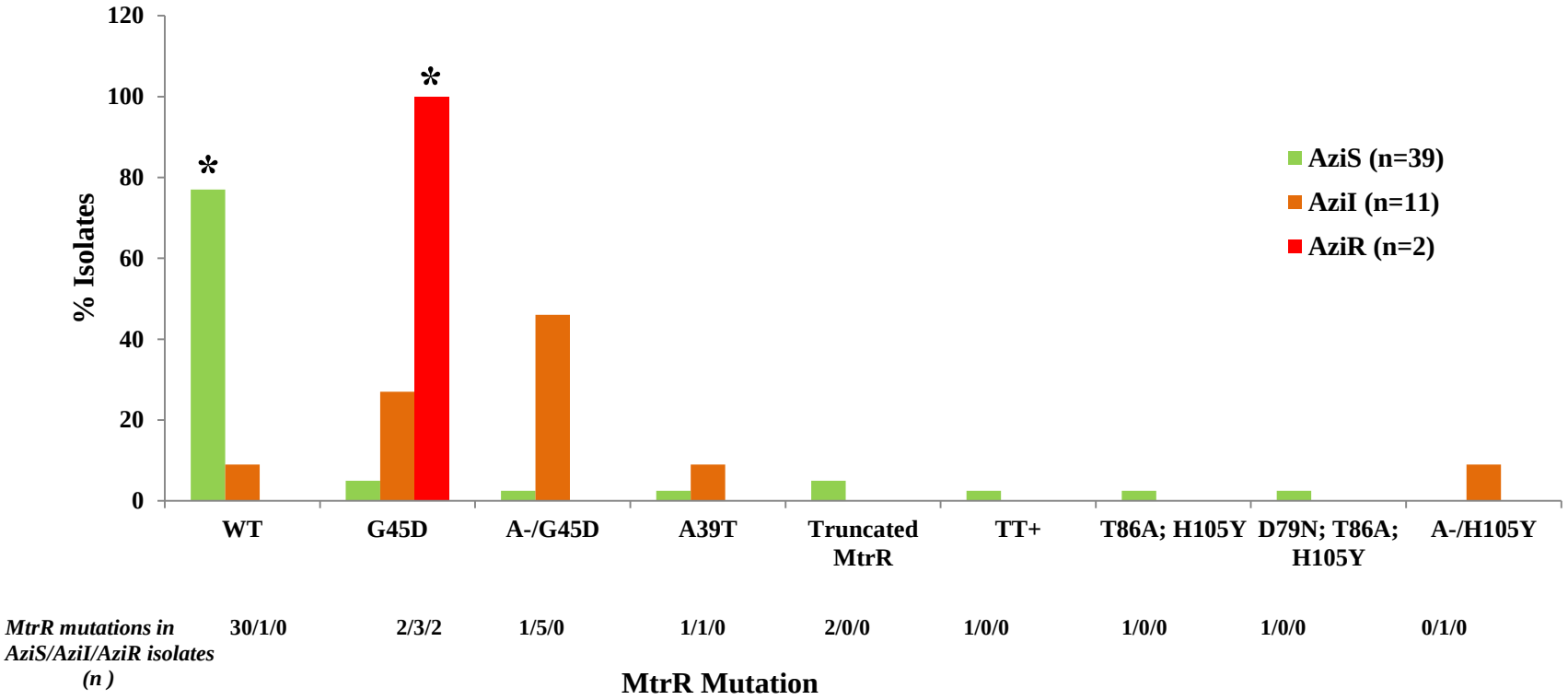

Fig. 3.15 Mutations in MtrR and its promoter in 52 *N. gonorrhoeae* isolates with different azithromycin susceptibility. Amino acid substitutions in MtrR and its promoter were determined in azithromycin susceptible (AziS), azithromycin intermediately susceptible (AziI) and azithromycin resistant (AziR) isolates.
*: P value < 0.05 was considered statistically significant.

Table 3.4 GyrA and ParC QRDR mutation patterns in 41 *N. gonorrhoeae*

Mutation Pattern				n (%)	MIC (mg/L)
GyrA		ParC			
S91	D95	S87	S88		
F	G	R	-	5 (12.2)	0.5[a]-8.0[*]
F	N	-	P	1 (2.4)	4.0
F	N	R	P	1 (2.4)	0.5[a]
-	-	-	-	34 (83)	0.002-0.008

-: No mutation on these residues.
a: Intermediate susceptibility
*: P value<0.05 (MIC=2-8 mg/L).

3.3.4 Analysis of Genes implicated in Penicillin Resistance

High levels (96%) of penicillin susceptibility of gonococci were observed in this study. This is contrary to recent reports on penicillin susceptibility of *N. gonorrhoeae* from Canada and worldwide (CDC 2009; Cole et al 2010; Martin et al 2011; Allen et al 2011; WHO-WPR 2012; Starnino et al 2012). In view of low levels of penicillin resistance, molecular determinants of penicillin resistance were also investigated in this study.

i) *penA* (PBP2) Analysis

PBP2 pattern IX was significantly associated with isolates which were both penicillin intermediately susceptible (Pen^I, MIC=1) (34/67, 51%; P<0.0001) and penicillin resistant (Pen^R, MIC ≥ 2mg/L) isolates (10/15, 67%; P<0.0001) (Table 3.5). Pattern I was significantly associated with penicillin susceptible (Pen^S) isolates (38/64, 59%; P<0.0001) while pattern XXII were predominant in Pen^S (15/64, 23%) and Pen^I (18/67, 27%) isolates (Fig 3.16). The *N. gonorrhoeae* isolate with pattern XXXIV (Pen MIC=0.5 mg/L) was present in a Pen^S category.

ii) *porB* (PorB) Analysis

In PorB, a combination of aspartic acid (D) and asparagine (N) substitutions at G120 and A121 was significantly (21/67, 31%; P=0.0016) associated with Pen^I isolates (Table 3.6). Pen^R was significantly associated (P<0.0001with charged amino acid substitutions at residue 120 and 121; G120K and A121D (9/15, 60%). Sixty three isolates with WT PorB were mostly present in Pen^S (48/64, 75%; P<0.0001) and Pen^I (15/67; 22%) categories (Fig 3.17).

iii) *ponA* (PBP1) Analysis

The L421P amino acid substitution in *N. gonorrhoeae* PBP1 was significantly associated with Pen^I (n=59/67, 88%; P< 0.001) and Pen^R (n=14/15, 93%; P<0.001, data not shown).

Table 3.5 Mutation patterns of PBP2 in 146 *N. gonorrhoeae* isolates with different penicillin susceptibility phenotypes

PBP2 pattern[a]	n	Penicillin Category[b]		
		Pen^S n (%)	Pen^I n (%)	Pen^R n (%)
IX	50 (34)	6 (12)	34 (51)*	10 (67)*
I	41 (28)	38 (59)*	3 (4)	0 (0)
XXII	35 (24)	15 (23)	18 (27)	2 (13)
II	7 (5)	0 (0)	6(9)	1 (7)[c]
XII	6 (3.5)	2 (3)	2 (3)	2 (13)[c]
XIV	2 (1.4)	0 (0)	2 (3)	0 (0)
V	2 (1.4)	0 (0)	2(2)	0 (0)
WT[d]	2 (1.4)	2 (3)	0 (0)	0 (0)
XXXIV	1(0.7)	1(2)	0 (0)	0 (0)
Total	146	64 (44)	67 (46)	15 (10)

a: Mutation patterns were numbered as they have been previously referenced in the literature (Whiley et al, 2007a; Ohnishi et al 2011b).
b: Penicillin category: Pen^S: Pen susceptible (MIC=0.008-0.5mg/L); Pen^I: Pen intermediate (MIC=1.0 mg/L); Pen^R: Pen resistant (MIC≥2; PPNG included).
c: Includes PPNG.
d: WT: wild type using wild type PBP2 sequence M32091 (Spratt 1988).
*: P value<0.05 is considered statistically significant.

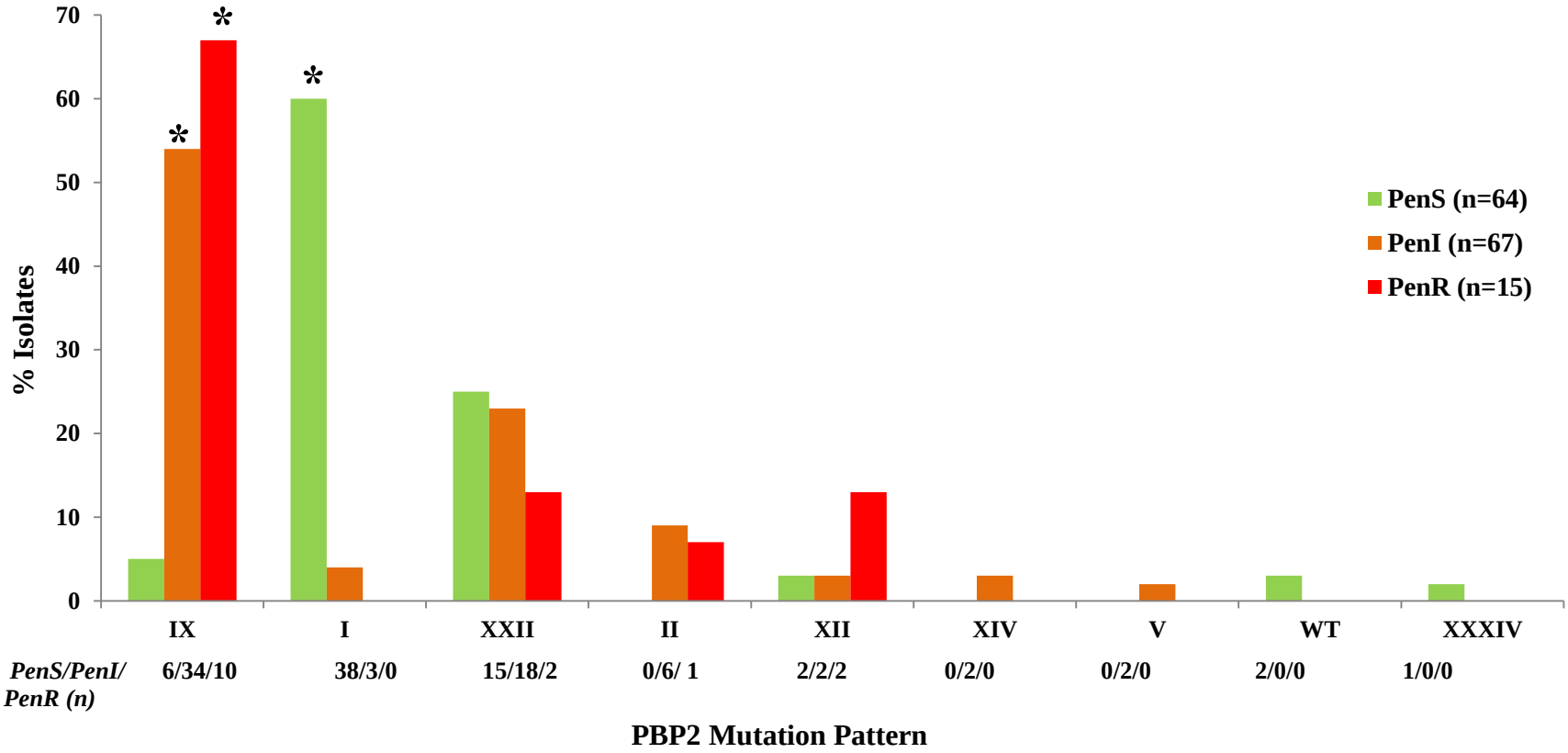

Fig. 3.16 PBP2 mutation patterns in 146 *N. gonorrhoeae* isolates with different penicillin susceptibility. Amino acid substitutions between AA340 to AA575 were determined. PenS: penicillin susceptible (MIC=0.008-0.5mg/L); PenI: penicillin intermediate (MIC=1.0 mg/L); PenR: penicillin resistant (MIC≥2; PPNG were also included).
*: P value < 0.05 was considered statistically significant.

Table 3.6 Mutation patterns in PorB in 146 *N. gonorrhoeae* isolates with different penicillin susceptibility phenotypes

Pattern number	PorB mutation	n	Penicillin Category[a]		
			Pen^S n (%)	Pen^I n (%)	Pen^R n (%)
1	WT[b]	63 (43)	48 (75)*	15 (22)	0 (0)
2	G120D;A121N	28 (19)	5 (8)	21 (31)*	0 (0)
3	G120K;A121D	21 (14)	5 (8)	7 (10)	9 (60)*
4	G120N;A121N	11 (7.5)	3 (5)	8 (12)	0 (0)
5	G120K;A121N	6 (4)	1 (2)	5 (7)	0 (0)
6	G120D;A121D	5 (3.5)	0 (0)	3 (4)	2 (13)
7	A121S;N122K	5 (3.5	0 (0)	4 (6)	1 (7)[c]
8	A121G	2 (1.4)	2 (3)	0 (0)	0 (0)
9	G120D	2 (1.4)	0 (0)	0 (0)	0 (0)
10	G120K	1 (0.7)	0 (0)	1(1.5)	0 (0)
11	G120K;A121G	1 (0.7)	0 (0)	0 (0)	1(7)
12	G120R;A121D	1 (0.7)	0 (0)	1 (1.5)	0 (0)
	Total	146	64 (44)	67 (46)	15 (10)

a: Penicillin category: Pen^S, Pen^I & Pen^R: See Table 3.7.
b: WT: wild type using *porB* sequence M21289 (Carbonetti et al 1987).
c: Includes PPNG.
*: P value<0.05 is considered statistically significant.

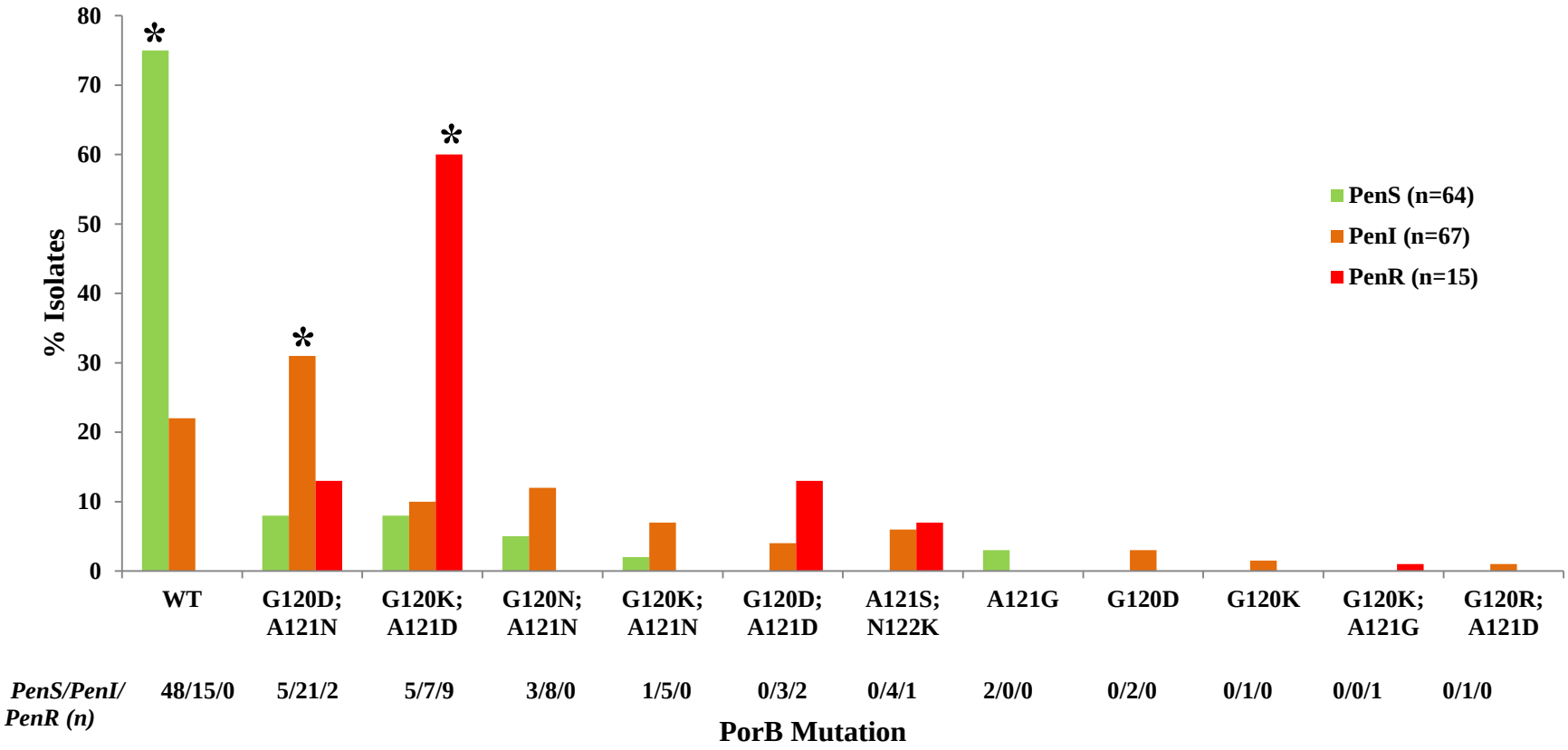

Fig. 3.17 PorB mutation patterns in 146 *N. gonorrhoeae* isolates with different penicillin susceptibility. Amino acid substitutions at G120 and A121 were determined in PenS, PenI and PenR isolates.
*: P value < 0.05 was considered statistically significant.

iv) *mtrR* (MtrR and its promoter) Analysis

A G45D substitution in the DNA binding domain of the MtrR was significantly associated with Pen^I (55%, 37/67; P<0.0001) and Pen^R (9/15, 60%; P=0.0005) isolates (Table 3.7). A-;G45D substitutions, in the *mtrR* promoter and DNA binding domain was also significantly associated with Pen^I (25%, 17/67; P=0.03) category. Wild type *mtrR* were associated with Pen^S (55%, 35/64; P<0.0001) isolates (Fig 3.18).

3.3.5 *penA*, *mtrR* and *porB* mutation Combinations Detected in *N. gonorrhoeae* Isolates Analyzed for Cephalosporin and Penicillin Susceptibility

A total of 50 mutation patterns; PBP2 (*penA*)/MtrR (*mtrR*)/PorB (*porB*) were observed in 146 isolates tested based on a combination of individual mutations in PBP2/MtrR/PorB (Table 3.8). Isolates from ceftriaxone group 2 (n=23) had 18 mutation patterns whereas 39 mutation patterns were found in 123 isolates from ceftriaxone susceptibility group 1. None of the patterns had significant association with ceftriaxone susceptibility group 2 or isolates with higher ceftriaxone MICs (MIC≥0.03 mg/L). Pattern I/WT//WT was significantly associated with ceftriaxone (33/123, 26.8%; P=0.01) and cefixime (33/122, 27%; P=0.01) susceptibility groups 1. Pattern IX/G45D/G120K;A121D (8/24, 33.3%) was significantly associated (P<0.0001) with cefixime susceptibility group 2 (Table 3.8).

Isolates from Pen^S (n=64) and Pen^I (n=67) categories had 25 and 30 PBP2/MtrR/PorB mutation patterns (Table 3.9). Penicillin resistant (n=15) isolates were classified into 6 PBP2/MtrR/PorB mutation patterns. Pen^S isolates were significantly related to pattern I/WT/WT (30/64, 47%; P=0.0001). Pen^I was significantly related to patterns IX/G45D/G120D;A121N (12/67, 18%; P=0.0012) IX/G45D/WT (9/67, 13.4; P=0.025). Patterns IX/G45D/G120K; A121D (7/15, 47%; P=0.007) and IX/G45D/G120D;A121D (2/15, 13.3%; P=0.04) had significant association with Pen^R isolates (Table 3.9).

Table 3.7 Mutation patterns of *mtrR N. gonorrhoeae* isolates associated with different penicillin susceptibility phenotypes

Pattern number	MtrR mutation	n	Penicillin Category[a]		
			Pen^S n (%)	Pen^I n (%)	Pen^R n (%)
1	WT[b]	37 (25)	35 (55)*	2 (3)	0
2	G45D	55 (38)	9 (14)	37 (55)*	9 (60)*
3	A-;G45D	25 (17)	6 (9)	17 (25)*	2 (13.0)
4	A-;H105Y	10 (7)	2 (3)	5 (7.5)	3(20)[c]
5	Truncated MtrR[d]	4 (3)	4 (6)	0	0
6	A39T	4 (3)	3 (4.5)	0	1(6.7)[c]
7	T86A;H105Y	3 (2)	1(1.5)	2 (3)	0
8	A40D;D79N;T86A;H105Y	3 (2)	0	3 (4.5)	0
9	D79N;T86A;H105Y	2 (1.4)	1(1.5)	1(1.5)	0
10	A39T;H105Y	1 (0.7)	1(1.5)	0	0
11	A-	1(0.7)	1(1.5)	0	0
12	TT+	1(0.7)	1(1.5)	0	0
	Total	146	64 (44)	67 (46)	15 (10)

a: Penicillin category: Pen^S, Pen^I & Pen^R: See Table 3.7.
b: wild type using *mtrR* sequence Z25796 (Pan & Spratt 1994).
c: Includes PPNG.
d: MtrR coding sequence truncated at amino acid position 136.
*: P value<0.05 is considered statistically significant.

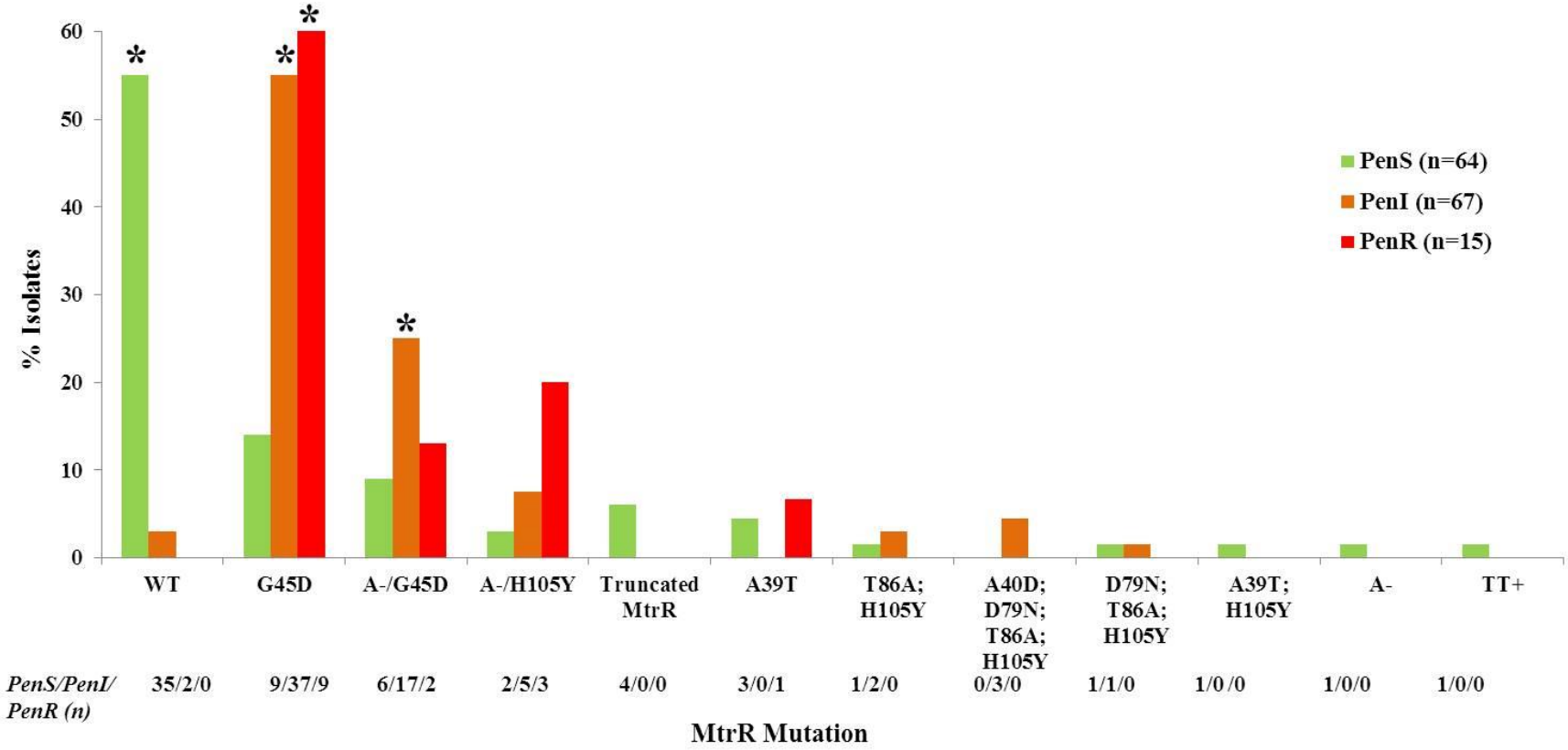

Fig. 3.18 Mutations in MtrR and its promoter in 146 *N. gonorrhoeae* isolates with different penicillin susceptibility. Amino acid substitutions in MtrR and its promoter were determined in PenS, PenI and PenR isolates.
*: P value < 0.05 was considered statistically significant.

Table 3.8 Mutation pattern combination of PBP2/ MtrR /PorB associated with ceftriaxone susceptibility in 146 *N. gonorrhoeae* isolates

Pattern numbers	PBP2/MtrR/PorB mutation pattern	n (%)	Ceftriaxone susceptibility groups[a]		Cefixime susceptibility groups[b]	
			1 n (%)	2 n (%)	1 n (%)	2 n (%)
A	I/WT/WT	33 (22.6)	**33[*] (26.8)**	0 (0)	**33[*] (27)**	0 (0)
B	IX/G45D/G120K;A121D	14 (10)	9 (7.3)	5 (22)	6 (5)	**8[*] (33.3)**
C	IX/ G45D/G120D;A121N	11 (8)	9 (7.3)	2 (8.7)	8 (6.5)	3 (12.5)
D	IX/G45D/ G120D;A121D	4 (2.7)	3 (2.4)	1 (4.4)	4 (3.3)	0 (0)
E	IX/G45D/WT	10 (7)	9 (7.3)	1 (4.4)	9 (7.4)	1 (4.2)
F	IX/ A-;H105Y/G120K;A121D	2 (1.4)	1 (0.8)	1 (4.4)	1 (0.8)	1 (4.2)
G	XXII/A-,G45D/G120N;A121N	8 (5.5)	7 (6)	1 (4.4)	8 (6.5)	0 (0)
H	XXII/G45D/G120D;A121N	4 (2.7)	3 (2.4)	1 (4.4)	4 (3.3)	0 (0)
I	I/G45D/ WT	3 (2)	3 (2.4)	0 (0)	1 (0.8)	2 (8.3)
J	I/ truncated MtrR/WT	3 (2)	3 (2.4)	0 (0)	2 (1.6)	1 (4.2)
K	II/G45D/A121S;N122K	2 (1.4)	2 (1.6)	0 (0)	2 (1.6)	0 (0)
L	II/A40D,D79N,T86A,H105Y/G120D	2 (1.4)	2 (1.6)	0 (0)	2 (1.6)	0 (0)
M	XXII/WT/WT	2 (1.4)	2 (1.6)	0 (0)	2 (1.6)	0 (0)
N	XXII/A-,G45D/G120D;A121N	9 (6)	9 (7.3)	0 (0)	9 (7.4)	0 (0)
O	XXII/A-,G45D/G120K;A121N	4 (3)	4 (3.3)	0 (0)	4 (3.3)	0 (0)
P	Other patterns[c]	35 (24)	24 (19.5)	11 (48)	27 (22)	8 (33.3)
	Total	146	123 (84)	23 (16)	122 (83.5)	24 (16.5)

a: Ceftriaxone susceptibility groups: 1: MIC range =0.0005-0.016 mg/L); 2: MIC range =0.03-0.06 mg/L.
b: Cefixime susceptibility groups: 1: MIC range =0.0005-0.016 mg/L); 2: MIC range =0.03-0.125 mg/L.
c: 35 isolates were with single unique combination of PBP2/MtrR/PorB.
*: P value<0.05 is considered statistically significant.

Table 3.9 Mutation pattern combination of PBP2/ MtrR /PorB associated with penicillin susceptibility in 146 *N . gonorrhoeae* isolates

Pattern numbers	PBP2/MtrR/PorB mutation pattern	n (%)	Isolates with different mutation patterns in penicillin categories		
			Pen^S n (%)	Pen^I n (%)	Pen^R n (%)
A	I/WT/ /WT	32 (22)	**30 (47)***	2 (3)	0
B	IX/G45D/G120K;A121D	14 (10)	2 (3)	5 (7.4)	**7 (47)***
C	IX/G45D/G120D;A121N	12 (8)	0 (0)	**12 (18)***	0(0)
D	IX/G45D/G120D;A121D	4 (2.7)	0 (0)	2 (3)	**2 (13.3)***
E	IX/G45D/WT	10 (7)	1 (1.5)	**9 (13.4)***	0 (0)
F	IX/ A-, H105Y/G120K;A121D	2 (1.4)	1 (1.5)	0 (0)	1 (7)
G	XXII/ A-, G45D/G120N;A121N	8 (5.5)	3 (4.7)	5 (7.4)	0 (0)
H	XXII/ G45D/G120D;A121N	4 (2.7)	2 (3)	2 (3)	0 (0)
I	I/G45D/WT	3 (2)	2 (3)	1 (1.5)	0 (0)
J	I/ truncated MtrR/WT	3 (2)	3 (4.7)	0 (0)	0(0)
K	II/G45D/A121S;N122K	2 (1.4)	0 (0)	1 (1.5)	1 (7)
L	II/A40D;D79N;T86A;H105Y/G120D	2 (1.4)	0 (0)	2 (3)	0 (0)
M	XXII/WT/WT	2 (1.4)	2 (3)	0 (0)	0 (0)
N	XXII/G120D, A120N/A-;G45D	9 (6)	3 (4.7)	6 (9)	0 (0)
O	XXII/G120K, A120N/A-;G45D	4 (3)	0 (0)	2 (3)	2 (14)
P	Other patterns	35 (24)	15 (23)	18 (27)	2 (14)
	Total	146	64 (44)	67 (46)	15 (10)

a: Penicillin categories: See Table 3.5.

3.3.6 β-lactamase and *tetM* Plasmid Types

Only 4 PPNG were detected during study period (2003-2011) (Fig 3.4). 2 of the 4 PPNG were with African type plasmids, other 2 from 2009 and 2011 could not be typed because of non availability of the isolates from 2009-2011.

All (n=14) the TRNG observed in this study were Dutch type. TRNG prevalence was 5% (n=3) in 2004, 5.8% (n=3) in 2005, 12.7% (n=7) in 2006 and 2.2% (n=1) in 2007 (Fig 3.5).

3.4 Phylogeny of *Neisseria gonorrhoeae* Strains from Saskatchewan Based on *porB* typing, NG-MAST and MLST

3.4.1 *porB* DNA Sequence Analysis and Phylogeny

Analysis of *porB* sequences differentiated 320 *N. gonorrhoeae* isolates (2003-2008) from SK into 70 strain types (Table 3.10) with an average of 4.5 isolates per *porB* ST. The index of discrimination (ID) of *porB* typing for 320 isolates was 0.94. Of 70 STs, 35 *porB* STs were associated with two or more *N. gonorrhoeae* isolates whereas 35 *porB* STs comprised individual isolates. *porB* STs were assigned numbers consistent with the typing criteria used in the Dillon laboratory (Liao et al 2008, 2009). *porB* STs identified in this study were numbered as ST-40, ST-71 and consecutively from ST-108 to ST-175. Of the 320 *N. gonorrhoeae* isolates, 54.4% (n=174/320) were clustered under 6 STs [109 (n=57, 18.0%), 108 (n=39, 12%), 114 (n=22, 7%), 141 (n=22, 7%), 111 (n=21, 6.6%) and 120 (n=13, 4.0%)].

Isolates susceptible to all the tested antibiotics were significantly associated with ST 109 (n=46/104, 44%; P<0.0001) (Table 3.10). CMTR gonococaal isolates had significant presence in STs; 108 (n=30/181, 17%; P=0.001), 114 (n=19/181, 10.5%; P=0.007) and 120 (n=12/181, 6.6%; P=0.017). CMRNG and TRNG were significantly associated to ST 111 (n=9/13, 69%; P<0.0001) and ST 130 (10/13, 77%; P=0.0001).

Table 3.10 70 *porB* strain types with resistance phenotypes in 320 *N. gonorrhoeae* isolates (2003-2008)

***porB* ST**	**n**	**Susce-ptible**	**CM TR**	**CMR NG**	**TR NG**	**Other resistance phenotypes**						
						PP NG	**Cip^{R}**	**Azi^{R}**	**PPNG/ CMTR**	**TRNG/ Cip^{R}**	**CMTR/ Cip^{R}**	**CMTR/ Azi^{R}**
109	**57**	**46***	**11**	**-**	**-**	**-**	**-**	**-**	**-**		**-**	**-**
108	**39**	**6**	**30***	**2**	**1**	**-**	**-**	**-**	**-**	**-**	**-**	**-**
114	**22**	**3**	**19***	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**
141	**22**	**5**	**17**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**
111	**21**	**1**	**11**	**9***	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**
120	**13**	**1**	**12***	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**
146	12	6	6	-	-	-	-	-	-	-	-	-
130	11	1	-	-	10*	-	-	-	-	-	-	-
134	8	5	3	-	-	-	-	-	-	-	-	-
131	7	1	6	-	-	-	-	-	-	-	-	-
132	7	1	6	-	-	-	-	-	-	-	-	-
138	5	2	3	-	-	-	-	-	-	-	-	-
110	4	3	1	-	-	-	-	-	-	-	-	-
113	4	3	1	-	-	-	-	-	-	-	-	-
122	4	4	-	-	-	-	-	-	-	-	-	-
157	4	1	3	-	-	-	-	-	-	-	-	-
159	4	-	4	-	-	-	-	-	-	-	-	-
40	3	-	3	-	-	-	-	-	-	-	-	-
117	3	2	1	-	-	-	-	-	-	-	-	-
118	3	1	2	-	-	-	-	-	-	-	-	-
129	3	2	1	-	-	-	-	-	-	-	-	-
148	3	-	3	-	-	-	-	-	-	-	-	-
116	2	1	1	-	-	-	-	-	-	-	-	-
119	2	1	1	-	-	-	-	-	-	-	-	-
128	2	2	-	-	-	-	-	-	-	-	-	-
144	2	2	-	-	-	-	-	-	-	-	-	-
147	2	1	-	-	-	-	1	-	-	-	-	-
153	2	-	2	-	-	-	-	-	-	-	-	-
154	2	-	1	-	1	-	-	-	-	-	-	-
160	2	-	2	-	-	-	-	-	-	-	-	-
161	2	-	1	-	-	-	-	-	-	-	1	-
162	2	-	2	-	-	-	-	-	-	-	-	-
167	2	-	2	-	-	-	-	-	-	-	-	-
172	2	2	-	-	-	-	-	-	-	-	-	-
175	2	-	2	-	-	-	-	-	-	-	-	-
Others	35	4	21	2	1	1	1	1	1	1	1	1
Total (n=70)	**320**	**104**	**181**	**13**	**13**	**1**	**2**	**1**	**1**	**1**	**2**	**1**

porB STs in bold constitutes 54.4% (n=174/320) of the typed isolates
*: P value<0.05 is considered statistically significant

Multiple sequence alignment was done to determine differences between all the 320 *porB* [765 base pairs (bp)] DNA sequences used in typing. *porB* sequences with ⩽1% bp (7 bp) differences from the predominant *porB* ST were grouped together and the group was named after the ST with highest number of isolates (Table 3.11). On the basis of this classification, 12 *porB* (n=300, 93.8%) groups were identified with ID of 0.87. The major *porB* groups were 109, 108 and 111.

Group 109 was the largest *porB* group and 73 (22.8%) gonococcal isolates were clustered under this group (Table 3.11). This *porB* group in addition to *porB* ST 109 also had *porB* STs 110 (n=4), 157 (n=4), 144 (n=2), 147 (n=2), 167 (n=2), 135 (n=1) and 165 (n=1). The STs grouped as *porB* group 109 differed in 1-6 bp between them. Group 109 had a significant association with susceptible isolates (n=53/73, 72.6%, P<0.0001).

porB group 108 accounted for 72 (22.5%) isolates. Group 108 had *porB* STs 108 (n=30), 114 (n=22), 131 (n=7), 159 (n=4), 148 (n=3), 153 (n=2), 160 (n=2), 156 (n=1) and 142 (n=1) (Table 3.11). The difference in between STs clustered as *porB* group 108 ranged from 1 to 4 bp. Group 108 (n=57/181, 80.28%) was significantly (P<0.0001) associated with CMTR isolates.

porB group 111 (n=34, 10.6%) comprised of *porB* STs 111 (n=21), 130 (n=11), 115 (n=1) and 145 (n=1) with differences of 1 to 6 bp. This group had significant (P<0.0001) associations with CMRG (n=9/13, 69.23%) and TRNG (10/13, 77%) isolates. Group 141 was also associated with CMTR isolates (23/181, 12.7%, P=0.016).

Isolates (n=104) susceptible to all the tested antibiotics were differentiated into 27 *porB* STs (Fig 3.19). Of the 104 susceptible *N. gonorrhoeae* isolates, 55.7% (n=58) were grouped under 3 STs; ST 109 (n=46, 44%), ST 108 (n=6, 5.7%) and ST 146 (n=6, 5.7%). Other predominant *porB* STs among susceptible isolates were, 141 (n=5, 4.8%) and 134 (n=5, 4.8%). Gonococcal isolates susceptible to all the antibiotics tested in this study had 7 *porB* ST groups (Fig 3.19). 51% (n=53) of the susceptible isolates were grouped under *porB* group 109.

Table 3.11 *porB* groups in *N. gonorrhoeae* isolates (2003-2008) from Saskatchewan for *porB* STs with ⩾2 isolates

porB Group[a]	n[b] (%)	Predominant ST (n)	STs differ by within ⩽ 1% bp in *porB*[c] (n)	Significant association with resistance phenotypes
109	73 (22.8)	109 (57)	110 (4), 157 (4), 144 (2), 147 (2), 167 (2), 135 (1), 165 (1)	Susceptible
108	72 (22.5)	108 (30)	114 (22), 131 (7), 159 (4), 148 (3), 153 (2), 160 (2), 142 (1), 156 (1)	CMTR
111	34 (10.6)	111 (21)	130 (11), 115 (1), 145 (1)	CMRNG, TRNG
141	29 (9)	141(22)	154 (2), 166 (1), 168 (1), 169 (1), 171 (1), 173(1)	CMTR
132	18 (6)	132 (7)	122 (4), 119 (2), 161(2), 124 (1), 143 (1), 174 (1)	
113	13 (4)	113 (4)	118 (3), 129 (3), 116 (2), 112 (1)	
134	11 (3.4)	134 (8)	172 (2), 163 (1)	
40	6 (1.8)	40 (3)	136 (1), 140 (1), 164 (1)	
133	3 (1)	133 (1)	152 (1), 155 (1)	
162	3 (1)	162 (2)	158 (1)	
175	3 (1)	175 (2)	171 (1)	
71	2 (0.6)	71 (1)	139 (1)	
120	13 (4)	120 (13)		CMTR
146	12 (3.8)	146 (12)		
138	5 (1.6)	138 (5)		
117	3 (1.3)	117 (3)		
Total	300	191	109	

porB groups in bold constitute 56% (n=179/320) of the typed isolates
a: STs with ≤ 1% (n=1-7) base pair difference from predominant ST
b: 20 isolates appear as single ST
c: *porB* sequence used for *porB* typing was 765 bp long
d: P value < 0.05 was considered significant

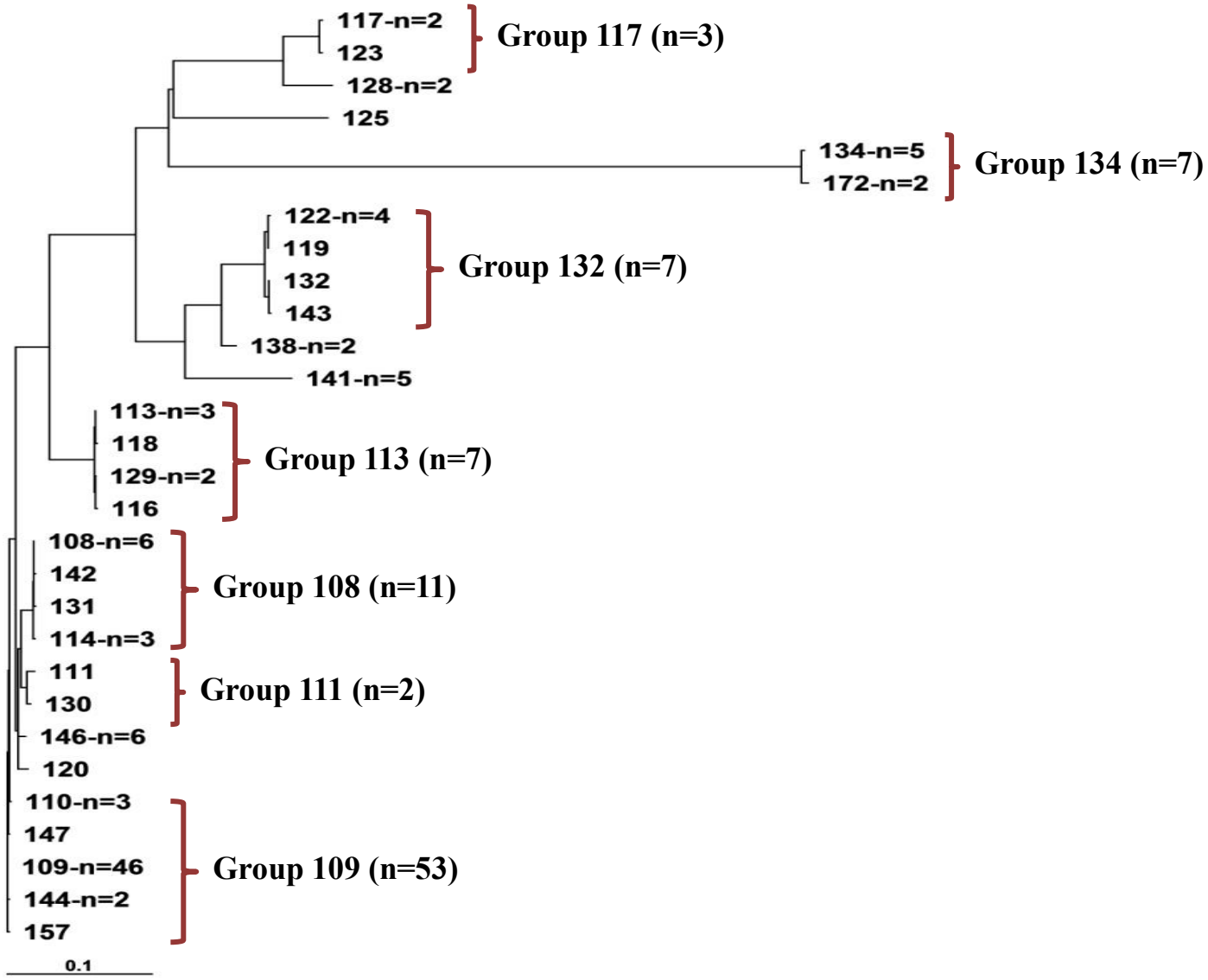

Fig. 3.19 *porB* STs associated with susceptible (n=104) *N. gonorrhoeae* isolates in Saskatchewan: 2003-2008.

The predominant *porB* STs: 109 (n=46, 44%), 108 (n=6, 5.7%), 146 (n=6, 5.7%), 141 (n=5, 4.8%) and 134 (n=5, 4.8%).

7 gonococcal *porB* ST groups were observed in susceptible isolates with ≤1% (1-7 bp) difference in amplified *porB* (765 bp) DNA sequence fragment.

51% (n=53) of the susceptible isolates were grouped under Group 109.

Phylogenetic analysis of *porB* STs showed clustering of CMRNG, TRNG and CMTR *N. gonorrhoeae* isolates. 4 STs were observed for CMRNG (n=13), ST-111(n=9, 69%), ST-108 (n=2, 15%), ST-137 (n=1, 7.7%) and ST-164 (n=1, 7.7%) (Fig 3.20A). ST-111 and ST-108 had 15 bp (≈2%) differences in *porB.*

TRNG (n=13) were characterized as 4 *porB* STs; ST-130 (10/13, 77%), ST-108 (1/13, 7.7%), ST-154 (1/13, 7.7%) and ST-149 (1/13, 7.7) (Fig 3.20B). *porB* ST-130 (10/11, 91%) was almost exclusively associated with TRNG (Table 3.13). One TRNG/Cip^R isolate observed in this study was identified as *porB* ST-151. This ST-151 had more than 100bp difference compares to other *porB* STs observed as TRNG in this study (data not shown).

CMTR (n=181) *N. gonorrhoeae* isolates were resolved into 50 *porB* STs (Fig 3.21). Of these 50 *porB* strain types, 28 STs were associated with one isolate each. Of the 181 CMTR *N. gonorrhoeae* isolates, 55% (n=100) were grouped under 6 *porB* STs [ST-108 (n=30, 17%), ST 114 (n=19, 10.5%), ST 141 (n=17, 9.4%), ST 120 (n=12, 6.6%), ST 109 (n=11, 6%) and ST 111 (n=11, 6%)]. This analysis of *porB* sequences showed presence of 10 *porB* groups (≥5 isolates) in CMTR *N. gonorrhoeae* (Fig 3.21). 37% (n=67) and 13% (n=23) of CMTR *N. gonorrhoeae* isolates were grouped under two *porB* separate groups; 108 and 141.

Among the 10 predominant *porB* STs observed in this study, 3 *porB* STs; 109, 108 and 114 were present throughout the entire study period (2003-2008) (Fig 3.22). Isolates appeared as ST 109 were significantly higher in 2003 (24/60, 40%, P<0.0001) and 2004 (17/59, 28.8%, P=0.024) and decreased in prevalence over the study period to 4.8% (2/41) of isolates in 2008. ST 114 (10/22, 45.5%, P=0.0008) predominated in 2006. The incidence of ST 111 (9/21, 42.8%, P=0.0071) was higher in 2004. ST 108 was present through the study period but no significant association of this ST was observed with time period. Interestingly, *porB* STs 108 &114 were grouped together as *porB* group 108 and *porB* STs 111 and 130 were grouped under *porB* group 111.

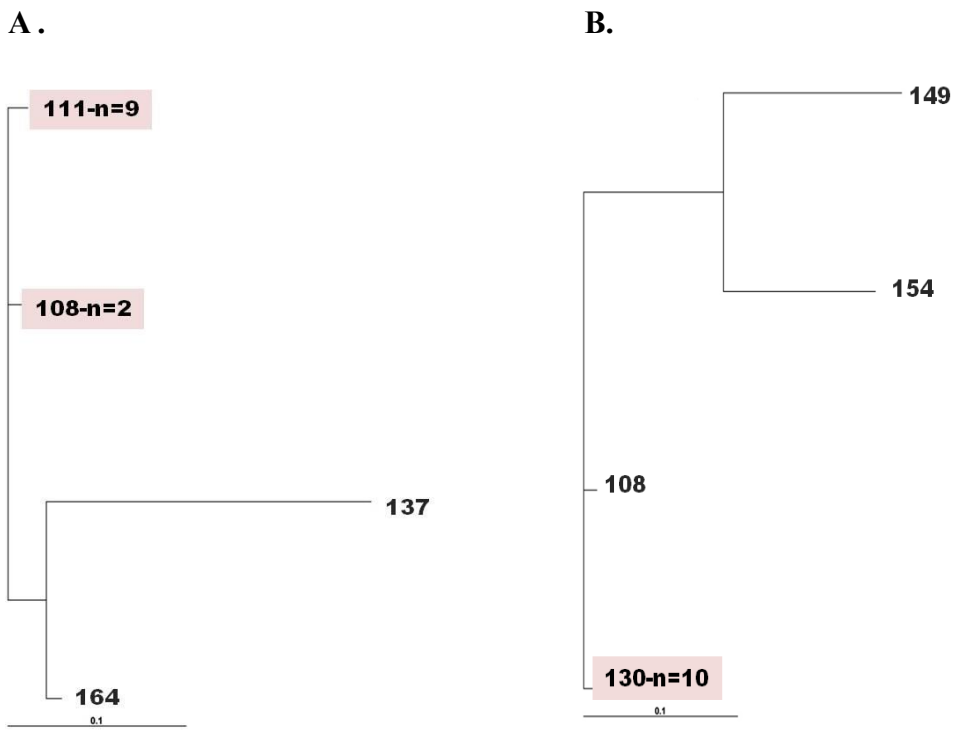

Fig. 3.20 *porB* STs associated with CMRNG (n=13) and TRNG (n=13) in Saskatchewan: 2003-2008

A. CMRNG, B. TRNG

A. 69% (9/13) and 15% (2/13) of CMRNG were grouped as *porB* ST-111 and 108. *porB* ST-111 and 108 had 15 bp (≈2%) difference.

B. 77% (10/13) TRNG clusterd under *porB* ST-130. The bp difference between ST-130 and other TRNG STs ranged from 12 bp (ST-108) to 153 bp (ST-149).

All the STs observed as CMRNG or TRNG were from different *porB* groups with >1% (> 7 bp) bp difference in the porB sequences.

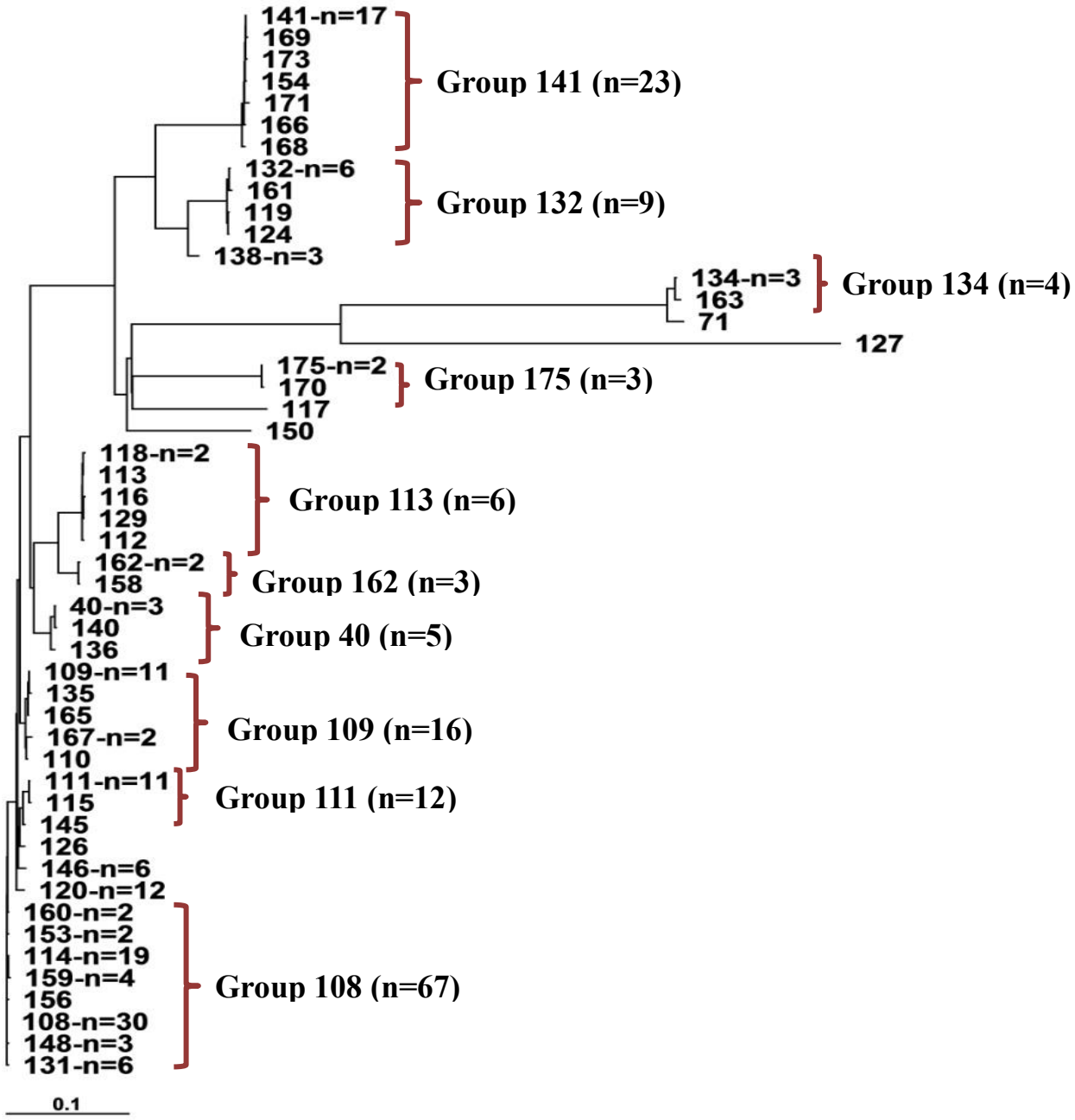

Fig. 3.21 *porB* STs associated with CMTR (n=181) *N. gonorrhoeae* isolates in Saskatchewan: 2003-2008.

The predominant *porB* STs: 108 (n=30, 17%), 114 (n=19, 10.5%), 141 (n=17, 9.4%), 120 (n=12, 6.6%), 109 (n=11, 6%) and 111 (n=11, 6%).

10 gonococcal *porB* groups were observed in CMTR gonococcal isolates.

37% (n=67) and 13% (n=23) of CMTR *N. gonorrhoeae* isolates were clustered under group 108 and 141.

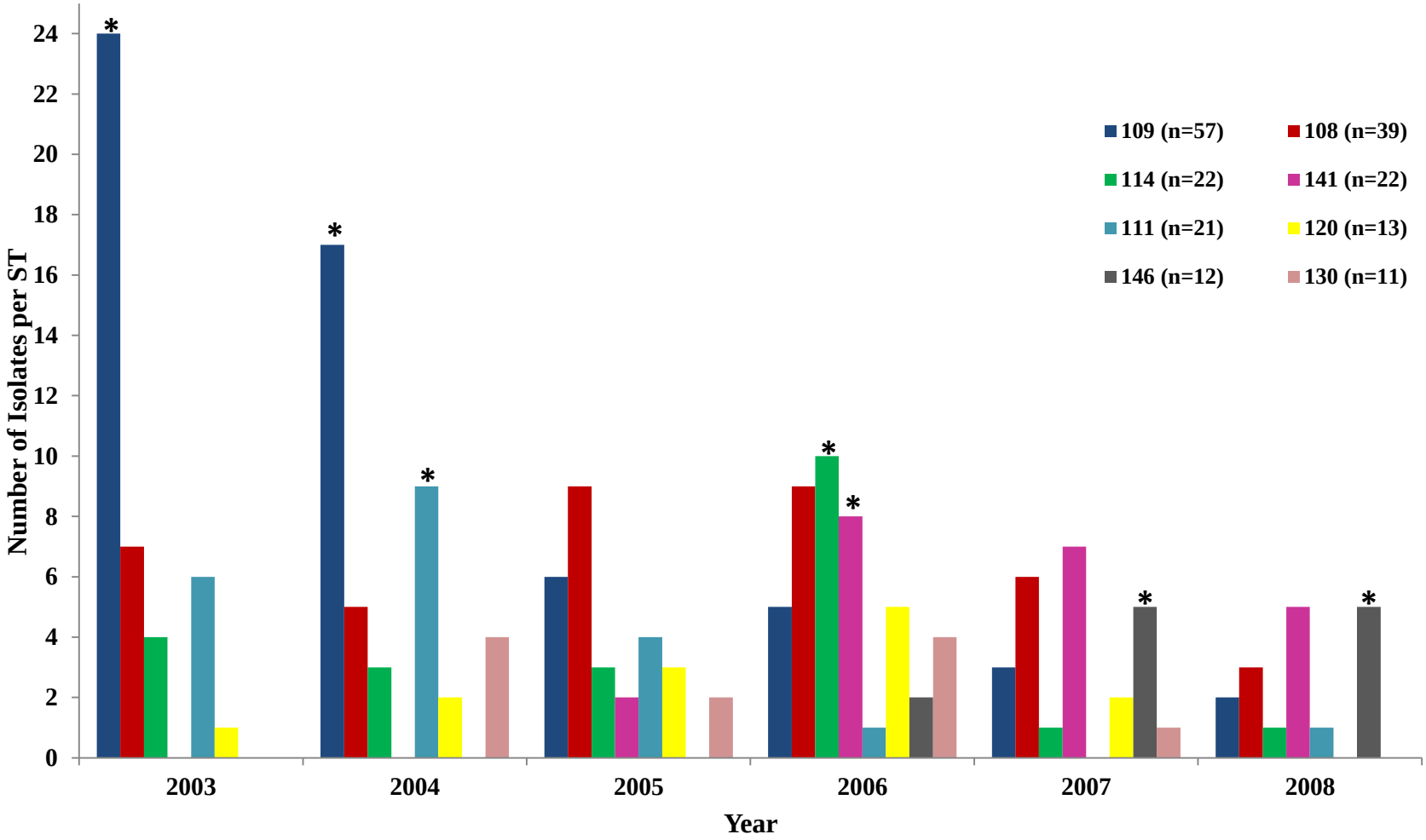

Fig. 3.22 Distribution of predominant (≥ 10 isolates) *porB* STs of *N. gonorrhoeae* in Saskatchewan (2003-2008). NG-MAST STs 25, 3655 and 3654 has shown declining trend in the prevalence after 2003, 2006 and 2004 respectively. *: P value < 0.05 was considered statistically significant.

3.4.2 NG-MAST Analysis and Phylogeny

NG-MAST resolved the 320 gonococcal isolates tested into 82 STs (Table 3.12) with an index of discrimination (ID) of 0.96. The average number of isolates per NG-MAST-ST was 3.9. 42 NG-MAST STs were associated with two or more *N. gonorrhoeae* isolates and 40 STs were associated with individual isolates. Of the 320 *N. gonorrhoeae* isolates, 55.6% (178/320) of the isolates grouped under 10 NG-MAST STs [25 (n=46, 14.4%), 3655 (n=23, 7.2%), 3654 (n=21, 6.5%), 3657 (n=15, 4.7%), 3672 (n=14, 4.4%), 921 (n=13, 4%), 3556 (n=13, 4%), 3711 (n=10, 3.1%) and 508 (n=10, 3.1%)] (Table 3.12).

Significant associations were observed between certain STs and antimicrobial resistance (AMR) profiles (Table 3.12). Susceptible isolates were significantly associated with STs 25 (40/46, 87%, P<0.0001). The CMTR phenotype was significantly associated with STs 3655 (22/23, 95.6%, P=0.0002), 3672 (12/14, 85.7, P=0.048) and 921 (12/13, 92.3%, P=0.0178). ST 3711 was exclusively associated with TRNG. The majority (9/13; 69.2%) of CMRNG were grouped as ST 3654 (9/21, 42.9%, P<0.0001).

The 82 STs identified were further grouped based NG-MAST STs which shared one identical *porB* or *tbpB* allele and which differed by ≤1% (⩽5bp difference in *porB* and ⩽4bp in *tbpB*) in the other allele (Table 3.13). On the basis of this classification, 29 NG-MAST groups were identified with ID of 0.93. The NG-MAST groups were named after the most predominant ST present in each group. Using this refined grouping scheme, the majority (53.4%, 171/320) of the isolates were grouped under 6 NG-MAST groups [25 (n=52, 16.3%), 3655 (n=35, 11%), 921 (n=27, 8.4%), 3654 (n=23, 7.2%), 3657 (n=20, 6.3%) and 3656 (n=14, 4.4%)]. NG-MAST STs 508 and 3655 had a one base pair difference in their *porB* allele (365: 2195) and were grouped as NG-MAST group 3655. STs 921 and 3714 differed by only one base pair in NG-MAST *porB* allele (356: 2242) and appeared as NG-MAST group 921 (Table 3.13).

Group 25 in addition to NG-MAST ST 25 also contained NG-MAST STs 50 (n=2), 70 (n=2) and 4674(n=2). STs clustered under NG-MAST group 25 had identical *tbpB* alleles and had 1 to 3 bp difference in *porB* (Table 3.13).

Table 3.12 82 NG-MAST strain types with resistance phenotypes in 320 *N. gonorrhoeae* isolates (2003-2008)

NG-MAST ST	n	Susce-ptible	CMTR	CMRNG	TRNG	Other resistance phenotypes						
						PPNG	Cip^R	Azi^R	PPNG/CMTR	TRNG/Cip^R	CMTR/Cip^R	CMTR/Azi^R
25	**46**	**40***	**6**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**
3655	**23**	**1**	**22***	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**
3654	**21**	**-**	**12**	**9***	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**
3657	**15**	**5**	**10**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**
3672	**14**	**2**	**12***	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**
3556	**13**	**6**	**7**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**
921	**13**	**1**	**12***	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**
3656	**13**	**4**	**8**	**1**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**
3711	**10**	**-**	**-**	**-**	**10***	**-**	**-**	**-**	**-**	**-**	**-**	**-**
508	**10**	**1**	**8**	**1**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**
3653	8	1	7	-	-	-	-	-	-	-	-	-
3671	7	4	3	-	-	-	-	-	-	-	-	-
3714	7	1	6	-	-	-	-	-	-	-	-	-
4585	6	5	1	-	-	-	-	-	-	-	-	-
210	5	2	3	-	-	-	-	-	-	-	-	-
2	4	2	2	-	-	-	-	-	-	-	-	-
12	4	3	1	-	-	-	-	-	-	-	-	-
1109	4	1	3	-	-	-	-	-	-	-	-	-
3676	4	3	1	-	-	-	-	-	-	-	-	-
3713	3	2	1	-	-	-	-	-	-	-	-	-
5	3	-	3	-	-	-	-	-	-	-	-	-
238	3	1	2	-	-	-	-	-	-	-	-	-
3652	3	-	3	-	-	-	-	-	-	-	-	-
3663	3	-	3	-	-	-	-	-	-	-	-	-
3670	3	2	1	-	-	-	-	-	-	-	-	-
4222	3	1	2	-	-	-	-	-	-	-	-	-
3660	2	-	2	-	-	-	-	-	-	-	-	-
3661	2	-	2	-	-	-	-	-	-	-	-	-
3662	2	-	2	-	-	-	-	-	-	-	-	-
3666	2	-	2	-	-	-	-	-	-	-	-	-
3669	2	-	2	-	-	-	-	-	-	-	-	-
3673	2	-	1	-	1	-	-	-	-	-	-	-
3677	2	-	2	-	-	-	-	-	-	-	-	-
3712	2	-	2	-	-	-	-	-	-	-	-	-
4228	2	1	1	-	-	-	-	-	-	-	-	-
4674	2	2	-	-	-	-	-	-	-	-	-	-

NG-MAST STs in bold constitute 55.6% (178/320) of the typed isolates
*: P value<0.05 is considered statistically significant

Table 3.12 continued…….

NG-MAST ST	n	Susce-ptible	CMTR	CMRNG	TRNG	Other resistance phenotypes						
						PPNG	Cip^R	Azi^R	PPNG/ CMTR	TRNG/ Cip^R	CMTR/ Cip^R	CMTR/ Azi^R
51	2	1	-	-	-	-	1	-	-	-	-	-
70	2	1	1	-	-	-	-	-	-	-	-	-
365	2	2	-	-	-	-	-	-	-	-	-	-
3116	2	-	1	-	-	-	-	-	-	-	1	-
3658	2	-	2	-	-	-	-	-	-	-	-	-
3659	2	-	2	-	-	-	-	-	-	-	-	-
Others	40	9	20	2	2	1	1	1	1	1	1	1
Total (n=82)	**320**	**104**	**181**	**13**	**13**	**1**	**2**	**1**	**1**	**1**	**2**	**1**

Table 3.13 NG-MAST groups in *N. gonorrhoeae* isolates (2003-2008) from Saskatchewan for NG-MAST STs with ⩾2 isolates

NG-MAST Group	n[a] (%)	Predominant ST (n)	STs which differ by ⩽1% for	
			porB[b] ST (n)	*tbpB*[c] ST (n)
25	**52 (16.3)**	**25 (46)**	**51 (2), 70 (2), 4674 (2)**	
3655	**35 (11)**	**3655 (23)**	**508[d] (10), 3658 (2)**	
921	**27 (8.4)**	**921 (13)**	**3662 (2), 3666 (2), 3675 (1), 3712 (2), 3714 (7)**	
3654	**23 (7.2)**	**3654 (21)**	**4516 (1), 4670 (1)**	
3657	**20 (6.3)**	**3657 (15)**	**3660 (2), 3677 (2), 3667 (1)**	
3656	**14 (4.4)**	**3656 (13)**		**4584 (1)**
3653	10 (3)	3653 (8)	3673 (2)	
12	10 (3)	12 (4)	238 (3), 3670 (3)	
3671	8 (2.5)	3671 (7)	3678 (1)	
210	8 (2.5)	210 (5)		3663 (3)
1109	7 (2.2)	1109 (4)	3659 (2), 4711 (1)	
5	5 (1.5)	5 (3)	3485 (1), 4673 (1)	
3669	3 (1)	3669 (2)	3664 (1)	
4228	3 (1)	4228 (2)	1650 (1)	
3116	3 (1%)	3116 (2)	3668 (1)	
4227	2 (0.6)	4227 (1)	4675 (1)	
225	2 (0.6)	225 (1)	4712 (1)	
64	2 (0.6)	64 (1)	359 (1)	
3672	14 (4.4)	3672 (14)		
3556	13(4)	3556 (13)		
3711	10 (3)	3711 (10)		
4585	6 (2)	4585(6)		
3676	4 (1.3)	3676(4)		
2	4 (1.3)	2 (4)		
3713	3 (1)	3713 (3)		
3652	3 (1)	3652 (3)		
4222	3 (1)	4222 (3)		
365	2 (0.6)	365 (2)		
3661	2 (0.6)	3661 (2)		
Total	298	233	61	4

NG-MAST groups in bold constitute 53.4% (171/320) of the typed isolates

a: 22 isolates appear as single STs

b: STs that share an identical *tbpB* allele but *porB* allele varies by ≤1%

c: STs that share an identical *porB* allele but *tbpB* allele varies by ≤1%

d: STs 508 and 3655 have ⩽1% difference in their *porB* (356:2195) alleles and have identical *tbpB* allele (28) ST 508 also shared *porB* (356) allele with 3566 and differed in *tbpB* allele (28:821) by ⩽1%. Since ST 3655 was more predominant compared to ST3656 therefore ST 508 was placed in group 3655

Group 3655 comprised of NG-MAST STs 3655(n=23), 508 (n=10) and 3658 (n=2) (Table 3.13). NG-MAST group 921 included NG-MAST STs 921 (n=13), 3662 (n=2), 3666 (n=2), 3675 (n=1), 3712 (n=2) and 3714 (n=7) (Table 3.13).

Susceptible isolates were significantly associated with NG-MAST group 25 (44/52, 84.6%, P<0.0001) (Table 3.14). NG-MAST groups; 3655 (32/35, 91.4%, P<0.0001), 921 (25/27, 92.6%, P=0.0002) and 3672 (12/14, 85.7%, P=0.048) were significantly associated with CMTR phenotype. CMRNG were significantly related to NG-MAST group 3654 (9/23, 39.1%, P<0.0001). NG-MAST group 3711 was exclusively associated with TRNG (P<0.0001) (Table 3.14).

Susceptible isolates (n=104) were resolved into 34 NG-MAST STs (Fig 3.23). More than 50% of susceptible isolates (56/104, 53.8%) appeared as 4 NG-MAST STs; 25 (n=40, 38.5%), 3556 (n=6, 5.7%), 3657(n=5, 4.8%) and 4585 (n=5, 4.8%). Forty-four (42.3%) susceptible isolates were clustered under group 25 (Fig 3.23).

NG-MAST grouped CMRNG into 4 STs, as observed with *porB* typing (Fig 3.24A). The majority of CMRNG (n=9/13, 69%) were clustered as NG-MAST ST-3654. TRNG were grouped under 4 NG-MAST STs (Fig 3.24B). NG-MAST ST-3711(n=10, 77%) was exclusively associated with TRNG.

N. gonorrhoeae CMTR isolates were grouped under 58 different NG-MAST STs (Fig 3.25). Of the 181 CMTR *N. gonorrhoeae* isolates, 57.4% (n=104) were grouped under 9 NG-MAST STs [3655 (n=22, 12.2%), 3654 (n=12, 6.6%), 3672 (n=12, 6.6%), 921 (n=12, 6.6%), 3657 (n=10, 5.5%), 508 (n=8, 4.4%), 3656 (n=8, 4.4%), 3653 (n=7, 3.9%) and 3653 (n=7, 3.9%). Twelve gonococcal NGMAST groups were observed in CMTR *N. gonorrhoeae* isolates. Twenty five (13.8%) CMTR isolates clustered under group 921. No clonality was detected with Cip or Azi resistant isolates.

Significant associations were observed between different STs and time periods of the study (Fig 3.26). Among the 10 predominant STs observed in this study, only 3 STs; 25, 3654 and 3655 were present throughout entire study period (2003-2008). The numbers of ST 25

Table 3.14 Major NG-MAST groups with resistance phenotypes in 320 *N. gonorrhoeae* isolates from Saskatchewan

NG-MAST Group	n	Susce-ptible	CMTR	CMRNG	TRNG	Other resistance phenotypes						
						PPNG	Cip^R	Azi^R	PPNG/ CMTR	TRNG/ Cip^R	CMTR/ Cip^R	CMTR/ Azi^R
25	**52**	**44***	**7**	**-**	**-**	**-**	**1**	**-**	**-**	**-**	**-**	**-**
3655	**35**	**2**	**32***	**1**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**
921	**27**	**2**	**25***	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**
3654	**23**	**1**	**13**	**9***	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**
3657	**20**	**8**	**12**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**
3656	**14**	**4**	**8**	**1**	**1**	**-**	**-**	**-**	**-**	**-**	**-**	**-**
3672	14	2	12*	-	-	-	-	-	-	-	-	-
3556	13	6	7	-	-	-	-	-	-	-	-	-
3711	10	-	-	-	10*	-	-	-	-	-	-	-
3653	10	1	8	-	1	-	-	-	-	-	-	-
12	10	6	4	-	-	-	-	-	-	-	-	-
3671	8	4	4	-	-	-	-	-	-	-	-	-
210	8	2	6	-	-	-	-	-	-	-	-	-
1109	7	1	6	-	-	-	-	-	-	-	-	-
4585	6	5	1	-	-	-	-	-	-	-	-	-
5	5	-	4	1	-	-	-	-	-	-	-	-
3676	4	3	1	-	-	-	-	-	-	-	-	-
2	4	2	2	-	-	-	-	-	-	-	-	-
3713	3	2	1	-	-	-	-	-	-	-	-	-
3116	3	-	1	-	-	-	-	-	-	-	2	-
3652	3	-	3	-	-	-	-	-	-	-	-	-
4222	3	1	2	-	-	-	-	-	-	-	-	-
3669	3	-	3	-	-	-	-	-	-	-	-	-
4228	3	1	2	-	-	-	-	-	-	-	-	-
64	2	-	-	-	-	-	-	1	-	-	-	1
3661	2	-	2	-	-	-	-	-	-	-	-	-
3677	2	-	2	-	-	-	-	-	-	-	-	-
4227	2	-	2	-	-	-	-	-	-	-	-	-
365	2	2	-	-	-	-	-	-	-	-	-	-
Others	22	5	11	1	1	1	1	-	1	1	-	-
Total	**320**	**104**	**181**	**13**	**13**	**1**	**2**	**1**	**1**	**1**	**2**	**1**

*: P value<0.05 is considered statistically significant

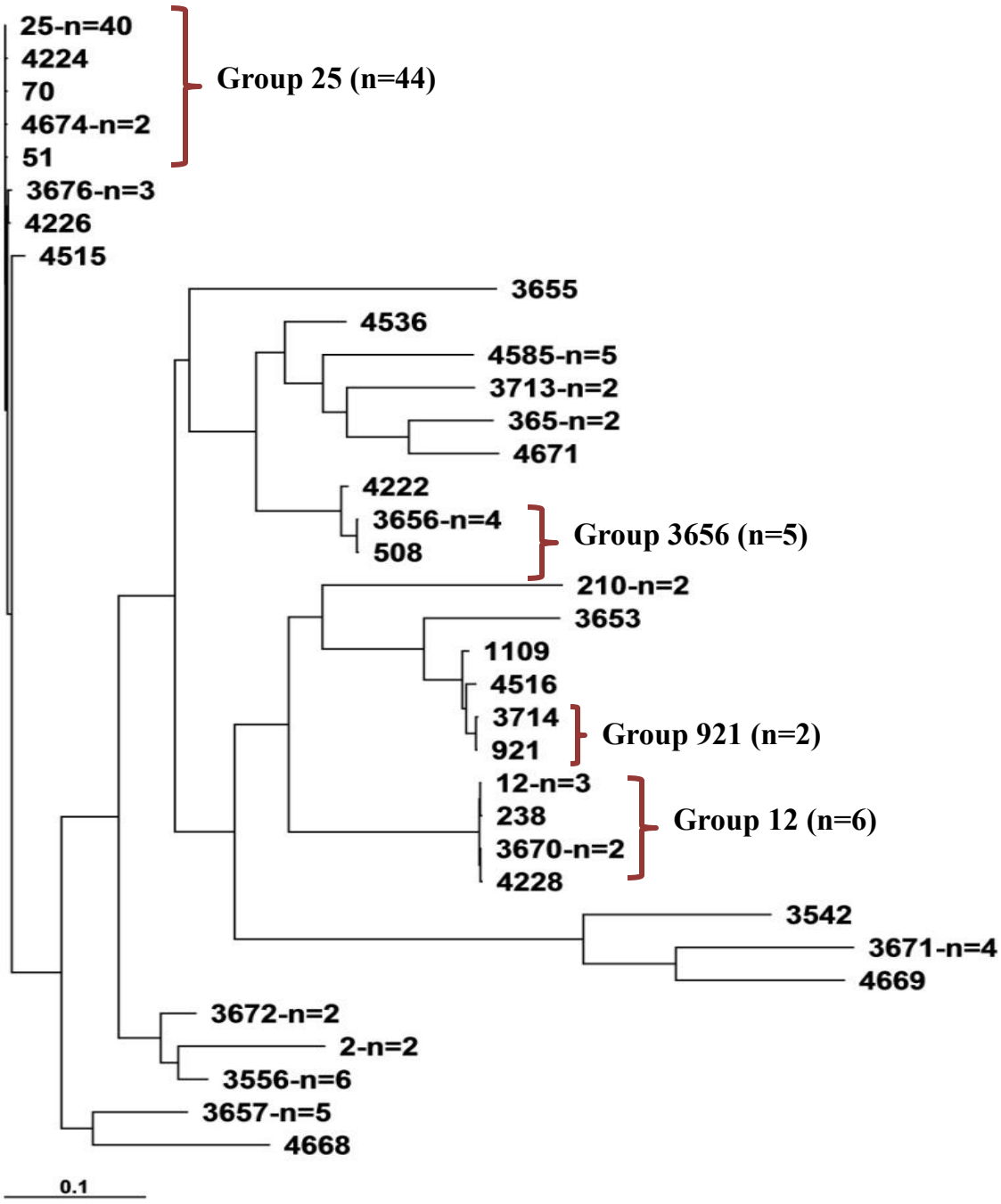

Fig. 3.23 NG-MAST STs associated with susceptible (n=104) *N. gonorrhoeae* isolates in Saskatchewan: 2003-2008.

The predominant NG-MAST STs: 25 (n=40, 38%), 3556 (n=6, 5.7%), 3657(n=5, 4.8%) and 4585 (n=5, 4.8%).

Four gonococcal NGMAST groups were observed in susceptible (n=104) *N. gonorrhoeae* isolates.

42.3% (n=44) of the susceptible isolates were clustered under group 25.

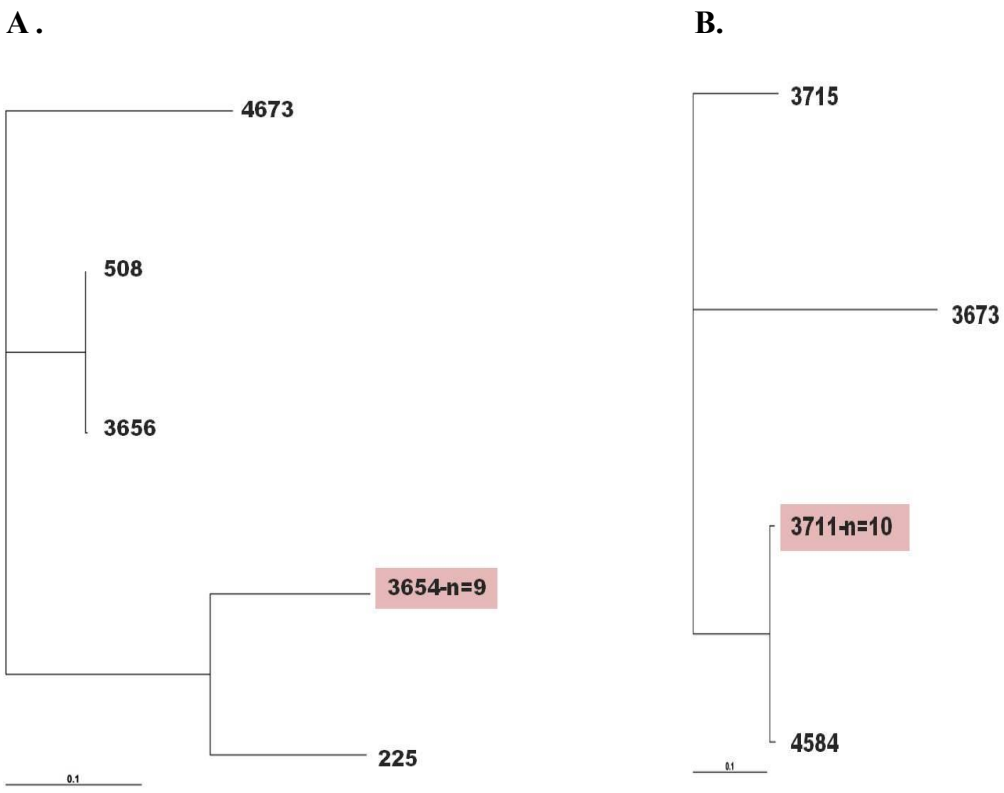

Fig. 3.24 NG-MAST STs associated with CMRNG (n=13) and TRNG (n=13) in Saskatchewan: 2003-2008

A. CMRNG, B. TRNG

A. 69% (n=9) CMRNG grouped as ST-3654.
B. 77% (n=10) TRNG were clustered under ST-3711 and this ST was exclusively associated with TRNG.

All CMRNG and TRNG isolates were from different NG-MAST groups.

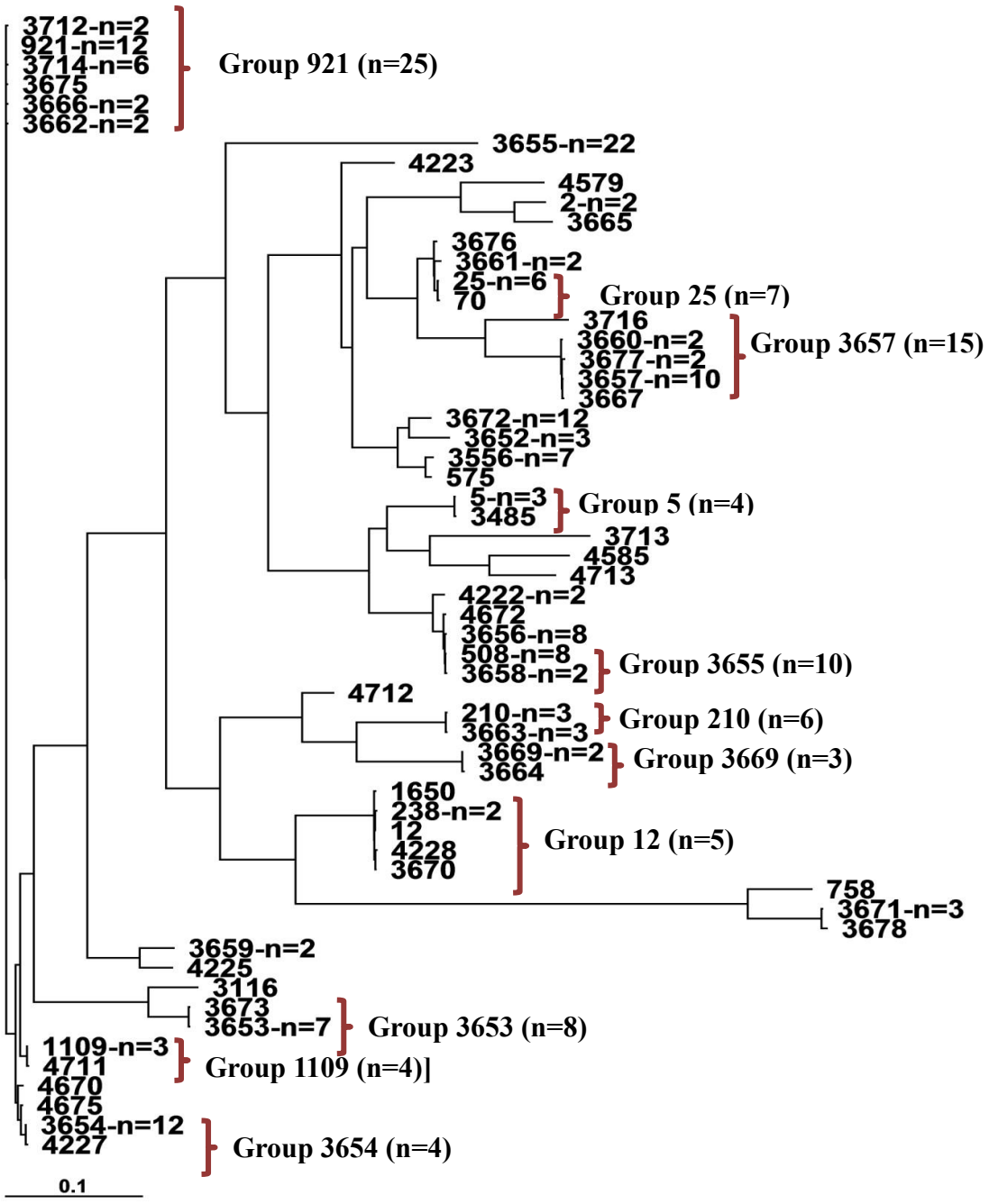

Fig. 3.25 NG-MAST STs associated with CMTR (n=181) *N. gonorrhoeae* isolates in Saskatchewan: 2003-2008.

The predominant NG-MAST STs: 3655 (n=22, 12%), 921 (n=12; 9.4%), 3654 (n=11, 6%), 3657 (n=10, 5.5%).

12 gonococcal NGMAST ST groups were observed in CMTR *N. gonorrhoeae* isolates. 13.8% of the CMTR isolates clustered under group 921.

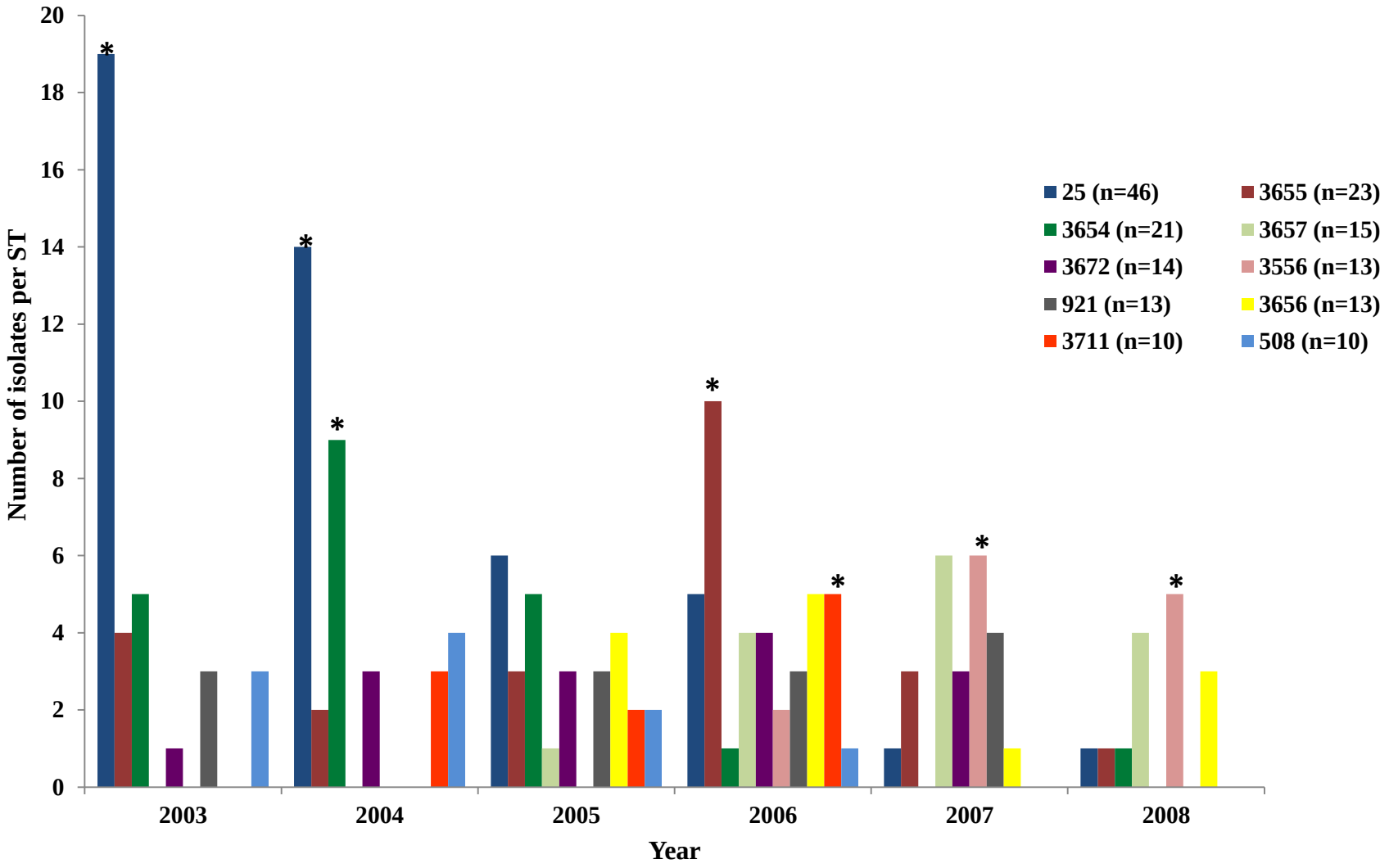

Fig. 3.26 Distribution of predominant (≥ 10 isolates) NG-MAST STs of *N. gonorrhoeae* in Saskatchewan (2003-2008).
NG-MAST STs 25, 3655 and 3654 has shown declining trend in the prevalence after 2003, 2006 and 2004 respectively.
*: P value < 0.05 was considered statistically significant.

isolates was significantly higher in 2003 (31.7%, 19/60, p<0.0001) and 2004 (23.7%, 14/59, p=0.032) and steadily declined in prevalence over the study period to 2.43% (1/41) of isolates in 2008. The prevalence of ST 3654 was significantly higher (15.3%, 9/59, p=0.0071) in 2004 and numbers decreased afterwards. ST 3655 (10/23, 43.5, p=0.0015) predominated in 2006. ST 3711 was observed in 2004 (5%, 3/59), 2005 (3.8%, 2/52) and 2006 (9%, 5/55) with a significant incidence in 2006 (p=0.017). ST 3556 had high numbers in 2007 (11.3%, 6/53, p=0.023) and 2008 (12.2%, 5/41, p=0.016).

It is important to mention that 7 of the 10 predominant STs observed in this study shares *porB* or (ST 508: ST3656) or *tbpB* (ST25: ST3657; ST3654: ST3653; ST508: ST3655) alleles.

3.4.3 MLST Analysis of *N. gonorrhoeae*

193 *N. gonorrhoeae* isolates (2005-2008) were resolved by MLST into 29 different STs (ST1 to ST29) (Fig 3.27) with an average of 6.7 isolates per MLST-ST. The index of discrimination of MLST for 193 isolates was 0.89. Most notably, 3 distinct STs were responsible for 52% *N. gonorrhoeae* isolates examined. ST-1 accounted for 23.6% (n=46) of the isolates, followed by ST-3 (15.9 %, n=31) and ST-2 (12.3%, n=24) (Fig 3.27). Seventy three isolates were resolved into 11 STs (ST-4, ST-5, ST-6, ST-8, ST-11, ST-14, ST-15, ST-16, ST-20, ST-21, and ST-25); these STs accounted from 1.5% (n=3) to 7.2% (n=14) of the isolates examined. Four STs (ST-9, ST-10, ST-24, and ST-29) were represented by two isolates each.

MLST resolved TRNG (n=9) from 2005 to 2008 in 3 STs; ST-20 (n=7), ST-25 (n=1) and ST-1 (n=1) (Fig 3.28). Six CMRNG (2005-2008) were differentiated into four STs; ST-1 (n=3), ST-3 (n=1), ST-9 (n=1), ST-25 (n=1) by MLST. 133 CMTR (2005-2008) isolates were resolved into 24 STs by MLST. The major STs among CMTR were ST-1 (n=41, 30.6%), ST-2 (n=16; 12%), ST-3 (n=26; 19%). 39 (2005-2008) susceptible isolates were clustered under 11 STs and predominant STs being ST-5 (n=9; 23%), ST-2 and 4 (n=7; 18%) and ST-3 (n=4; 10%).

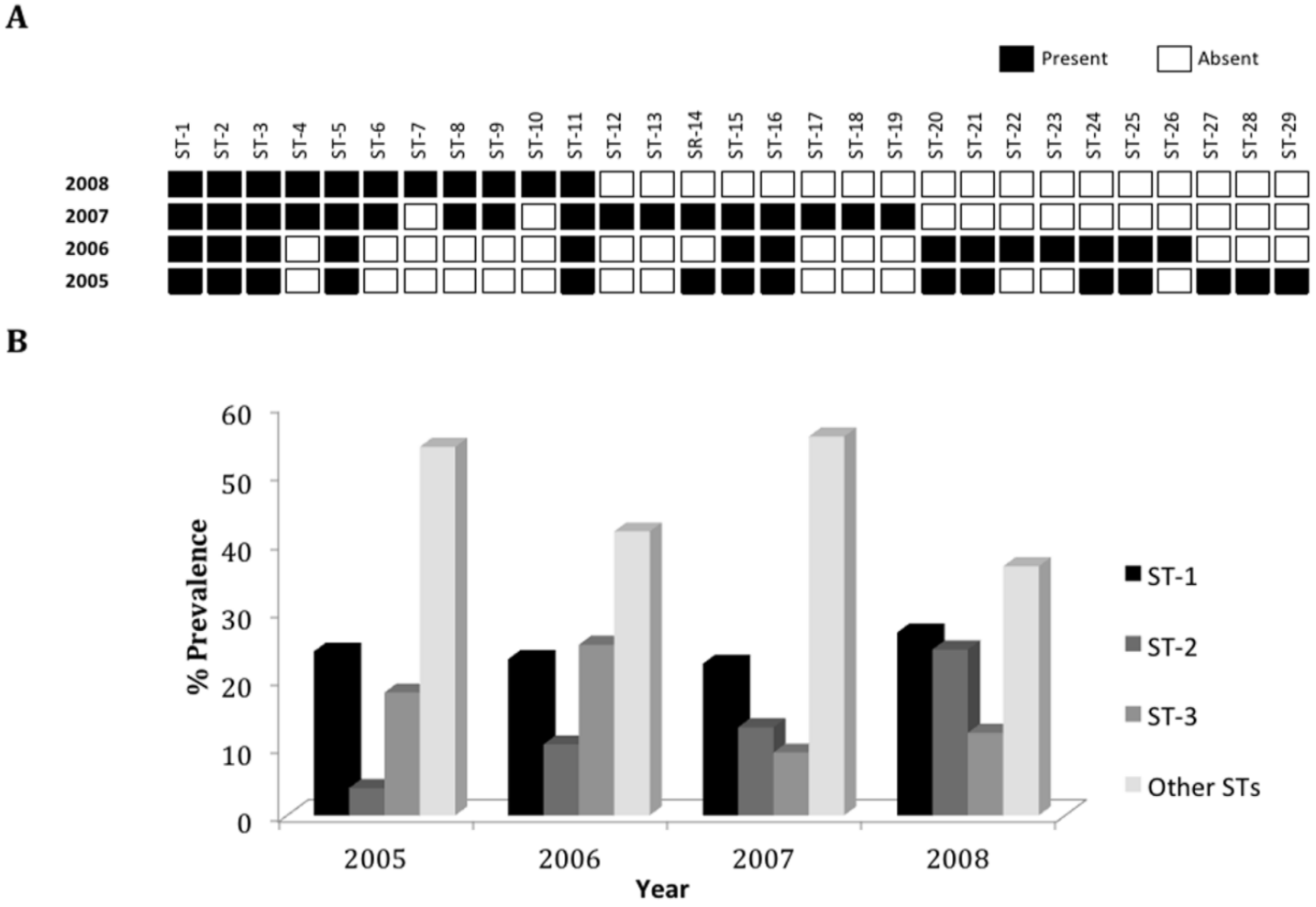

Fig. 3.27 Longitudinal distribution of *N. gonorrhoeae* strains in Saskatchewan on the basis of MLST: 2005-2008

A. Occurrence of the STs over a four-year period. Solid squares indicate the presence of certain STs, while open squares indicate their absence.
B. The prevalence rate of the most predominant circulating strains compared to the prevalence rate of all other STs identified during 2005-2008 in Saskatchewan.

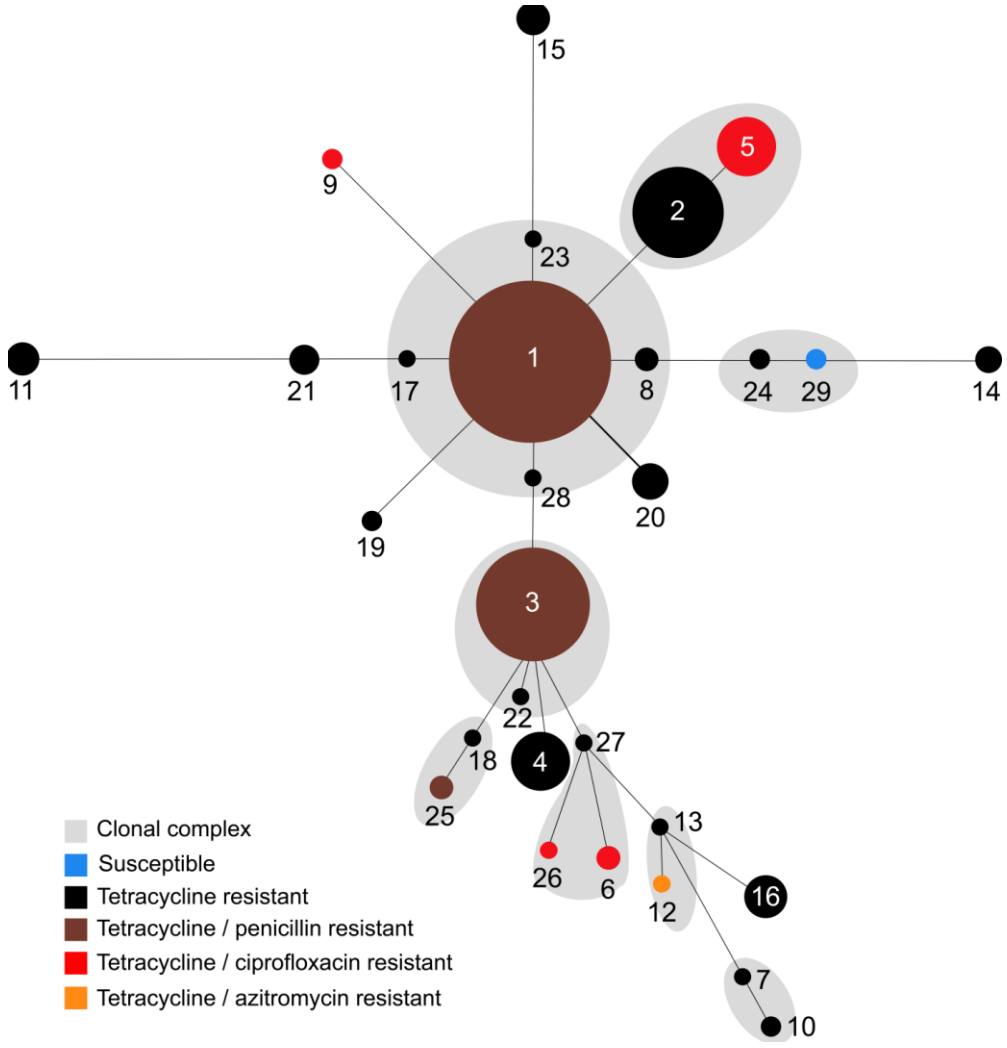

Fig. 3.28 Minimum spanning tree based on the multilocus sequence typing allelic profiles depicting antibiotic-resistant phenotype distribution across *N. gonorrhoeae* lineages and their clonal complexes in Saskatchewan: 2005-2008

Each circle represents unique ST. The number of isolates of each ST is directly proportional to the area of the circle. Blue circle represents the ST susceptible to all tested antibiotics, black indicates resistance to tetracycline alone, while brown color indicates resistance to penicillin and tetracycline, red resistance to ciprofloxacin and tetracycline and orange indicates resistance to azithromycin and tetracycline. Gray shadings represent the clonal complexes which consist of single locus variants.

Among Cip^R (n=5) isolates, two Cip^R isolates were ST-6 and remaining three were as single STs; 6, 9 and 26. The majority of CMRNG (5/6) were found in circulating strains ST-1 (n=3) and ST-3 (n=1). All Cip (n=5) and Azi (n=1) resistant isolates were found in sporadic sequence types. All these sporadic isolates were positioned on the edges of the minimum spanning tree (Fig 3.28).

3.4.4 Comparison of *porB* Typing, NG-MAST and MLST

Three typing methods used in this study, *porB*, NG-MAST and MLST were compared for 193 *N. gonorrhoeae* isolates (Table 3.15). NG-MAST, *porB* typing and MLST differentiated 193 isolates into 45, 43 and 29 STs, respectively. The indices of discrimination of the NG-MAST, *porB* typing, and MLST for these isolates were 0.97, 0.95, and 0.89.

All typing schemes resolved CMRNG (n=6) into 4 STs. TRNG (n=9) were differentiated into 3 STs by MLST and into 4 STs each by *porB* typing and NG-MAST. 133 CMTR gonococci isolates were resolved into 24, 30 and 32 STs on the basis of *porB* typing, NG-MAST and MLST, respectively. 39 susceptible *N. gonorrhoeae* were differentiated to 11 MLST STs, 17 *porB* STs and 18 NG-MAST STs.

Forty-six *N. gonorrhoeae* isolates clustered as ST-1 by MLST were differentiated into 15 and 16 STs through NG-MAST and *porB* typing (Fig 3.29). 16 NG-MAST observed in isolates clustered under MLST ST-1 had 13 *porB* and 3 *tbpB* sequence types. MLST ST-2 (n=24) were resolved as 7 NG-MAST STs and 9 *porB* STs. 31 isolates associated with MLST ST-3 were grouped as 4 NG-MAST STs and 6 *porB* STs. Only *porB* ST-141 and NG-MAST ST-3653 appeared in all the four years in isolates grouped under MLST ST-1. These observations were also made with MLST ST-2 and ST-3. *porB* ST-141 and NG-MAST ST-3657 were present for all the four years and were MLST-2. In MLST-3, *porB* ST-108 and NG-MAST ST-3656 were detected for all the four years (2005-2008).

Table 3.15 Strain types detected by *porB*, NG-MAST and MLST in 193 *N. gonorrhoeae* isolates (2005-2008)

Phenotype	n	Number of STs detected		
		NGMAST	*porB*	MLST
Susceptible	39	18	17	11
CMRNG	6	4	4	4
TRNG	9	4	4	3
CMTR	133	32	30	24
Cip^R	2	2	2	2
Azi^R	1	1	1	1
Cip^R/TRNG	1	1	1	1
Cip^R/CMTR	2	2	2	2
Total	193	45	43	29

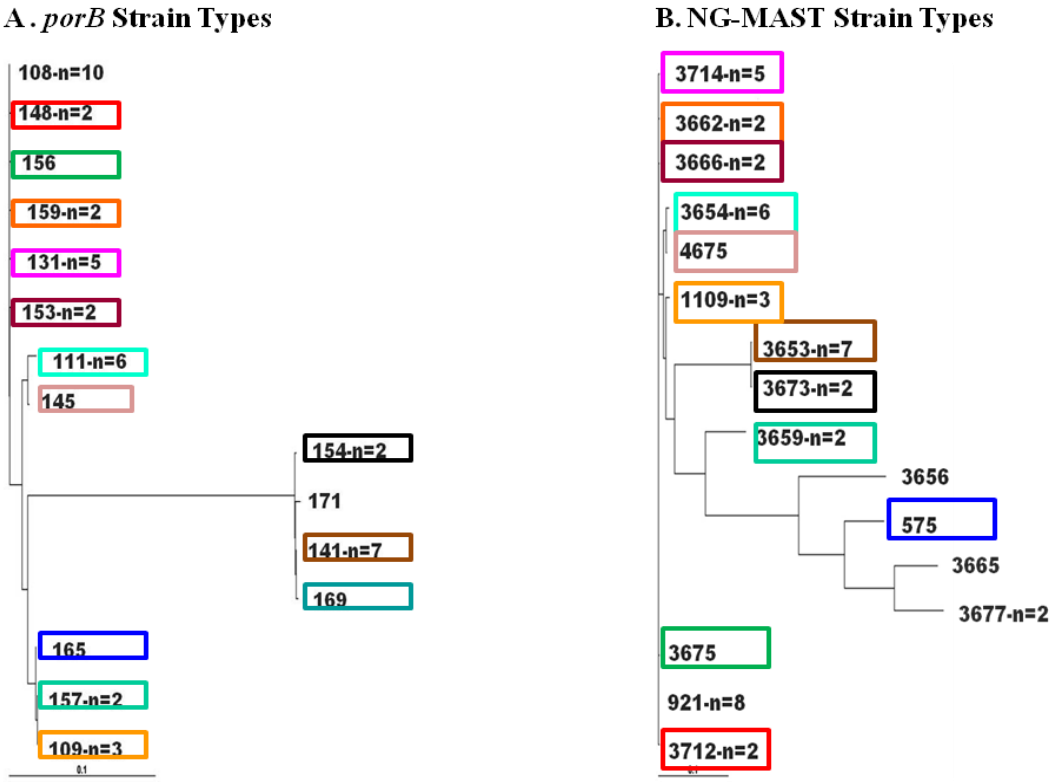

Fig. 3.29 Differentiation of *N. gonorrhoeae* (n=46) isolates clustered under MLST ST-1 by *porB* typing and NG-MAST

A. 46 isolates clustered as MLST ST-1 were resolved into 15 STs by *porB* typing.
B. MLST ST-1 was differentiated into 16 STs by NG-MAST.

porB and NG-MAST STs with same isolates are boxed similarly colored box i.e. *porB* STs 148 and 111 have same isolates as those of NG-MAST STs 3712 and 3659.

porB ST-108 (n=10) was resolved into 3 NG-MAST STs; 921 (n=8), 3665 (n=1) and 3677(n=1). *porB* ST-171 was NG-MAST ST-3677.

3.4.5 Association of Predominant NG-MAST and MLST STs with PBP2, MtrR and PorB Mutations

N. gonorrhoeae isolates (n=146) analyzed for molecular mechanisms of resistance were classified into 51 STs; 6 STs (86/146; 59%) comprised ≥ 5 isolates, 10 STs included 2-4 isolates, and 35 STs contained 1 isolate (Table 3.16). These STs carrying specific combinations of mutation patterns were significantly related to certain resistance phenotypes. Isolates with NGMAST-ST 25 (33/36, 92%) were associated (P<0.0001) with PBP2 (*penA*)/MtrR (*mtrR*)/PorB (*porB*) pattern I/WT/WT (Fig 3.30) and with antibiotic susceptibility. NG-MAST ST 3654 was associated (P<0.0001) with *penA/mtrR/porB* pattern IX/G45D/G120K;A121D (n=13/17) and CMRNG (n=7) and CMTR (n=6) isolates. Isolates with chromosomal resistance to tetracycline were significantly associated (P<0.0001) with several STs and *penA/mtrR/porB* patterns including: ST 3655 (XXII/A-;G45D/G120N;A121N - n=8/12), ST 921 (pattern IX/G45D/G120D;A121N - n=6/9), ST 508 (XXII/G45D/G120D;A121N - n=6/6), and ST 3656 (pattern XXII/A-; G45D/G120D;A121N – n=5/6). 24 isolates had higher cefixime MICs (0.03-0.06 mg/L) and included 17 STs with *penA* pattern IX (n=17) and *mtrR* G45D (n=16) and *porB* G120K, A121D (n=12) mutations. Seven of these isolates were associated (P<0.0001) with ST 3654 (pattern IX/G45D/G120K;A121D).

Table 3.16 Association of PBP2, MtrR and PorB mutations in major NG-MAST STs resistance phenotypes

NG-MAST ST (total)	n	Patterns			Resistance Phenotypes				
		penA	*mtrR*	*porB*	Susceptible	CMTR	CMRNG	TRNG	Higher ceftriaxone/cefixime MIC (⩾0.03 mg/L)
25 (36)	**33***	I	WT	WT	**33***	0	0	0	
	1	IX	G45D	G120D; A121D	0	1	0	0	
	1	XXII	WT	WT	1	0	0	0	
	1	XXII	A-/G45D	WT	1	0	0	0	
3654 (17)	**3***	IX	G45D	G120D; A121D	0	1	2	0	
	13*	IX	G45D	G120K; A121D	0	**6***	**7***	0	3/**7***
	1	XXII	G45D	G120K; A121D	0	1	0	0	1/0
3655 (12)	**8***	XXII	A-/G45D	G120N; A121N	0	**8***	0	0	1/1
	1	XXII	A-/G45D	G120D; A121N	0	1	0	0	
	1	XXII	A-/G45D	G120K; A121N	0	1	0	0	
	1	XII	A-/G45D	G120D; A121N	0	1	0	0	
	1	IX	A-/G45D	G120N; A121N	0	1	0	0	
921 (9)	**6***	IX	G45D	G120D; A121N	0	**6***	0	0	1/1
	3	XXII	G45D	G120D; A121N	0	3	0	0	
508 (6)	**6***	XXII	G45D	G120D; A121N	0	**5***	1	0	
3656 (6)	**5***	XXII	A-/G45D	G120D; A121N	0	**4***	1	0	
	1	XXII	G45D	G120D; A121N	0	1	0	0	
3714 (4)	3*	IX	G45D	G120D; A121N	0	**3***	0	0	2/2
	1	IX	G45D	G120K; A121N	0	1	0	0	
3657 (4)	3*	I	Truncated MtrR	WT	3*	0	0	0	1/1
	1	I	A39T	WT	1	0	0	0	1/0
1109 (3)	3*	IX	G45D	WT	0	**3***	0	0	
Total	97				39	47	11	0	

In total 14 isolates (9 susceptible, 3 CMTR, 2 TRNG) were grouped as 2 isolates per ST. Thirty five total 35 isolates (3 susceptible, 21 CMTR, 1 TRNG, 1 PPNG, 2 CMRNG, 1PPNG/CMTR, 1 TRNG/Cip^R, 2 CMTR/Cip^R, 2 Cip^R and 1 Azi^R isolates) were grouped as single isolate per ST.
*: P value <0.05 considered significant.

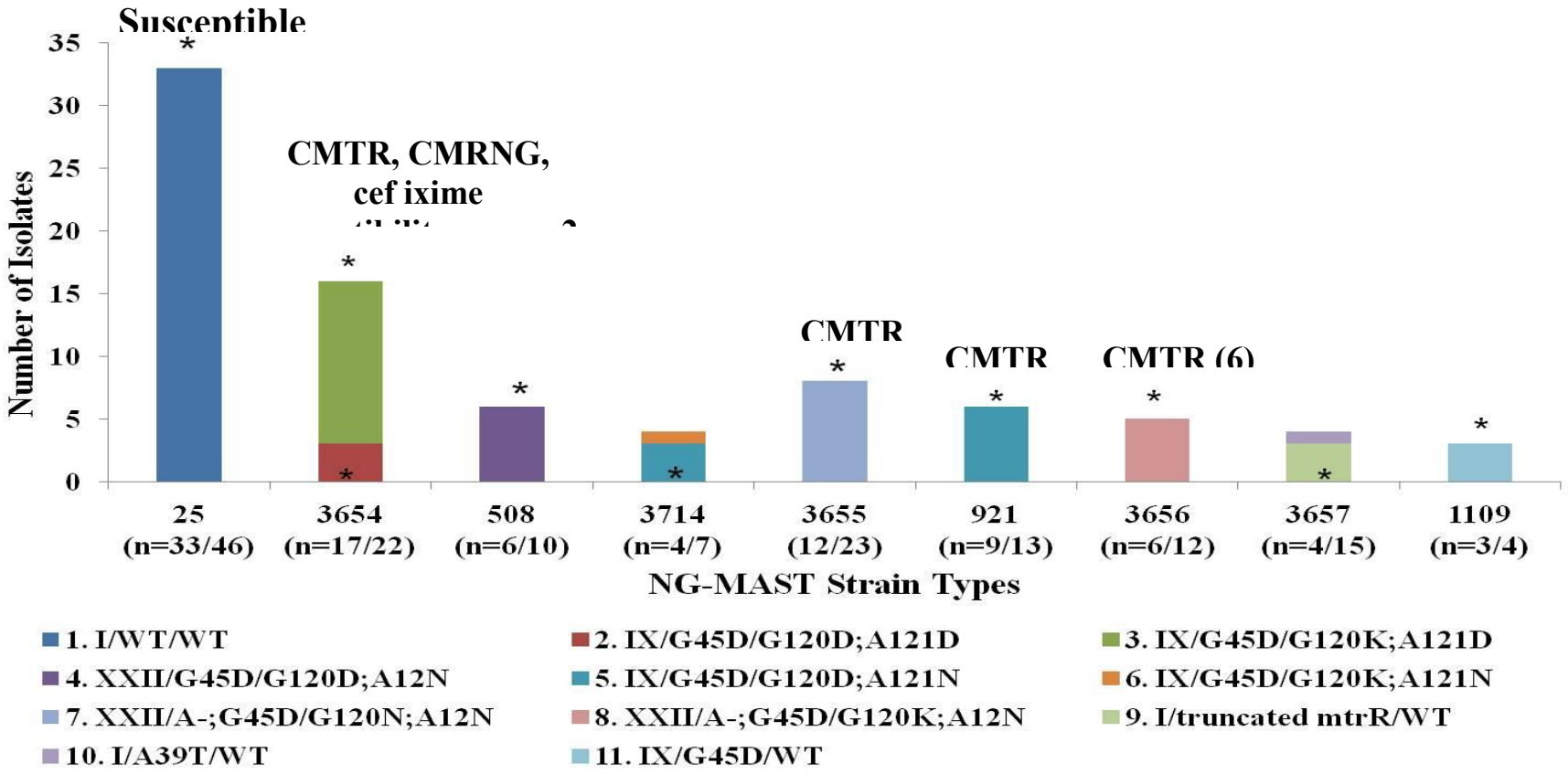

Fig. 3.30 Significant (P<0.05) associations between NG-MAST strain types and combination of mutations in PBP2, MtrR and PorB.

Figures in parenthesis indicate the mutation pattern numbers with which significant associations were observed with specific antibiotic resistance phenotype in a specific NG-MAST ST.

CHAPTER FOUR

DISCUSSION

4.1 Prevalence of Antimicrobial Resistance in *Neisseria gonorrhoeae* in Saskatchewan

Results of this research indicate that *N. gonorrhoeae* isolates from SK were highly susceptible (>95%) to antibiotics currently recommended (ceftriaxone, cefixime, azithromycin & ciprofloxacin) and previously (penicillin & spectinomycin) recommended for the treatment of gonorrhea in Canada. Aggregate resistance to all the tested antibiotics detected in this study (2003-2011) was below 5% except for tetracycline. AMR prevalence in *N. gonorrhoeae* in SK is much lower compared to Canadian national resistance rates (Martin et al 2011) and rates detected in other Canadian provinces (Ota et al 2009; Plitt et al 2009; Allen et al 2011). This might be attributed to the fact that these provinces have larger populations and are major national transit, economic and immigration hubs compared to SK. Such activities introduce new infections to naive populations from distant places.

One of the challenges faced by the laboratories performing surveillance of antimicrobial resistance in *N. gonorrhoeae* is the shift from the use of cultures (required for antimicrobial susceptibility testing) to the NAATs for the diagnosis of gonorrhea. This has decreased the availability of bacterial cultures for antimicrobial susceptibility testing and may result in underestimations of AMR in *N. gonorrhoeae* (Dillon 2011). A few (n=8) isolates tested in this study were from the throat (n=5/427, 1.16%) and the rectum (n=3/427, 0.7%), common sites of infection in MSM. Antimicrobial susceptibility testing of lower number of *N. gonorrhoeae* isolates from MSM in this study may have also contributed to the underestimation of AMR in gonococcal isolates from SK. A higher prevalence of antimicrobial resistant *N. gonorrhoeae* strains has been reported in MSM (HPA 2008; Ota et al 2009; de Vries et al 2009; CDC 2009, 2011; Bola et al 2012). The proportion of *N. gonorrhoeae* isolates in MSM with elevated MICs (cefixime ≥0.25 mg/L; ceftriaxone≥0.125mg/L) of extended spectrum cephalosporins in the USA have increased from 0.2% to 4.7% (24 fold) for cefixime and 0.0% to 1.0% (10 fold) for ceftriaxone from 2006 through 2011 (Bolan et al 2012).

The majority of the resistant isolates in this study were from three major cities of the province, Saskatoon, Regina and Prince Albert. This can be attributed to the larger population of these cities which is also reflected in the higher number of isolates tested from these cities.

N. gonorrhoeae has developed resistance to every class of antimicrobial used to treat gonorrhea since the introduction of sulphonamides in the early 1940s and has resulted in reduced treatment options for gonococcal infections (Lewis 2010; Dillon 2011). The emergence of PPNG in 1976 ended the use of penicillin as a drug of choice for gonorrhea treatment (Percival 1976) and the development of chromosomal resistance to tetracycline resulted in the removal of this antibiotic from treatment guidelines (Reyan et al 1958). Emergence of significant resistance to quinolones, specifically to ciprofloxacin in different parts of the world, resulted in the withdrawal of this class of antibiotics from gonorrhea treatment guidelines (CDC 2006, 2007, PHAC 2006, 2008b, 2010a,b). After the spread of QRNG strains worldwide, extended spectrum cephalosporins (cefixime & ceftriaxone) became the antibiotics of choice for the treatment of gonococcal infections (Wang & Zhang 2007; PHAC 2006; CDC 2006, 2007). Recent reports on *N. gonorrhoeae* isolates with the reduced susceptibility or resistance to extended spectrum cephalosporins and gonorrhea treatment failures with extended spectrum cephalosporins has put a question mark on the longevity of this class of antibiotics as single dose treatment of gonorrhea (Tapsall et al 2009a; Lewis 2009; Bala et al 2010; Dillon 2011; Bolan et al 2012). Isolations of *N. gonorrhoeae* strains from Japan and Europe with high level resistance to cefixime, ceftriaxone and other antibiotics has initiated a broad discussion on the use of novel antimicrobials such as gentamicin and new antimicrobial combinations to treat gonorrhea (Golparian et al 2010, Ohinishi et al 2011; Unemo et al 2010; 2011a&b, 2012a&b; Cámara et al 2012).

In its new treatment guidelines, the Public Health Agency of Canada (PHAC, 2011c), recommends use of ceftriaxone (250 mg intramuscularly) or cefixime (800 mg orally) to treat gonococcal infections. Cefixime is not effective in treating pharyngeal gonorrhea, common in MSM (CDC 2012; Tapsall et al 2009b). Ceftriaxone is now being recommended as the preferred treatment for gonococcal infections in MSM. These treatment guidelines also recommend use of one gram single oral dose of azithromycin along with recommended doses of extended spectrum

cephalosporins as an empirical treatment to treat *Chlamydia* and gonococcl co-infections. It is important to note that PHAC still recommends quinolones (ciprofloxacin 500 mg or ofloaxcin 400 mg as single dose orally) as alternative treatment in those cases where quinolone susceptibility of gonococcal isolates is demonstrated through antimicrobial susceptibility testing or local resistance is under 5% with a test of cure can be done.

4.1.1 Absence of *N. gonorrhoeae* Isolates with Reduced Susceptibility to Cephalosporins

None of the 427 *N. gonorrhoeae* isolates tested from SK had reduced susceptibility (MIC=0.00025-0.25 mg/L) or resistance to cefixime and ceftriaxone. No drift towards higher MIC values was noted for oral (cefixime) or parentral (ceftriaxone) cephalosporins in the province. In Canada, none of the isolates tested for AMR between 2000 and 2009, were resistant to cefixime or ceftriaxone, but *N. gonorrhoeae* isolates with decreased susceptibility to cefixime (MIC 0.25 mg/L & 0.5 mg/L) and ceftriaxone (MIC 0.125 mg/L & 0.25 mg/L) were reported with a MIC shift to the right (Martin et al 2011). The proportion of *N. gonorrhoeae* isolates with an elevated MIC of cefixime (≥0.25 mg/L) and ceftriaxone (≥0.125mg/L) in the USA has increased by a factor of 17 (0.1% in 2006 to 1.7% in 2011) and 10 (0.05% in 2006 to 0.50% in 2011) between 2006 and 2011, respectively (Bolan et al 2012). A recent report from Ontario, Canada observed that 9.4% of tested *N. gonorrhoeae* isolates had reduced susceptibility to cefixime (MICs = 0.125–0.25 mg/L), or ceftriaxone (MICs = 0.032–0.125 mg/L) (Allen et al 2011).

The National Gonococcal Isolate Surveillance Project (GISP), USA has reported 20 isolates with decreased susceptibility to cefixime (MIC=0.5 mg/L) since 2000 (CDC 2009, 2011). In 2010, 88.9% of gonococcal isolates with decreased susceptibility to cefixime were recovered from MSM (CDC 2011). GISP has reported four isolates with decreased susceptibility to ceftriaxone (MIC of 0.5 mg/L) in the USA since 1987. No isolates with reduced susceptibility to ceftriaxone (MIC ≥ 0.5 mg/L) were observed in 2010 (CDC 2011). However; the percentage of isolates with elevated ceftriaxone MICs increased from 0.1% to 0.3% during 2000 and 2010 (CDC 2011) in the USA.

Reduced cephalosporin susceptibility and high level of resistance to extended spectrum cephalosporins (cefixime and ceftriaxone) among *N. gonorrhoeae* has been reported from Europe (Martin et al 2006; Chisholm et al 2010; Cole et al 2010). These isolates were recovered from Italy (Martin et al 2006), Spain (Vázquez et al 2007; Cámara et al 2012), Greece (Tzelepi et al 2008), the UK (HPA 2008, 2009; Chisholm et al 2010), Norway (Unemo et al 2010), Sweden (Golparian et al, 2010), Austria (Unemo et al, 2011a), France (Unemo et al 2012a) and Slovenia (Unemo et al, 2012b). In Europe, 5% of tested *N. gonorrhoeae* isolates in 2009 displayed decreased susceptibility (>0.125 mg/L) to cefixime (Cole et al 2011). No reduced susceptibility to ceftriaxone (>0.125 mg/L) in *N. gonorrhoeae* isolates was reported from Europe in 2009 (Cole et al 2011). In Portugal from 2004 through 2009, 4 isolates displayed decreased susceptibility to ceftriaxone (≥0.125mg/L ≤0.25 mg/L) (Florindo et al 2010).

Early reports on reduced susceptibility or resistance to extended spectrum cephalosporins in *N. gonorrhoeae* isolates had appeared from Western Pacific region especially Japan (Ameyama et al 2002; Tanaka et al 2002, 2006; Ito et al, 2004; Ohnishi et al 2011a) and Australia (Tapsall et al 2008, Tapsall 2009). Such gonococcal isolates have also been isolated from Thailand (Clendennen et al 1992a), the Philippines (Clendennen et al 1992b), Hong Kong SAR (Lo et al 2008), India (Bala et al 2007), Vietnam (Cao et al 2008), South Korea (Lee et al 2010) and China (Yang et al 2006; Liao et al 2011). *N. gonorrhoeae* isolates with cefixime and ceftriaxone MICs as high as 2 mg/L or more were reported in Japan and China (WHO 2006; Guoming et al 2000; Ohnishi et al 2011a). The proportion of gonococci with decreased susceptibility to ceftriaxone in Western Pacific countries/regions in 2010 ranged from 1.3% in Singapore to 55.8% in China (WHO-WPR 2012).

Limited data from Africa have not reported any isolates with reduced susceptibility and resistance to cefixime and ceftriaxone (Barry & Klausner 2009). Recently, Mehta et al (2011) had reported cefixime and ceftriaxone MIC creep in *N. gonorrhoeae* isolates from Kenya.

N. gonorrhoeae isolates tested for antimicrobial susceptibility in 11 Latin American countries during 2000 and 2009 were ceftriaxone susceptible with the exception of 6% of isolates tested in Manaus, Brazil, in 2007 that had ceftriaxone MICs >0.25 mg/L (Starnino et al 2012).

Gonorrhea treatment failures with extended spectrum cephalosporins have been primarily reported with the use of oral cephalosporins such as cefixime (Barry & Klausner 2009). Initial reports of treatment failures with cefixime appeared from Japan (Ameyama et al 2002; Ito et al 2004; Tanaka et al 2002, 2006; Takahata et al 2006) followed by Hong Kong (Lo et al 2008) and then from various European countries (Golparian et al 2010, Unemo et al, 2010, 2011a). Treatment failures with ceftriaxone, a parental cephalosporin has been reported from Australia (Tapsall et al, 2009b), Japan (Muratani et al, 2008; Ohnishi et al 2011a,b), Sweden (Golparian et al 2010, Unemo et al 2011b) and Slovenia (Unemo et al 2012b). These reports indicate that the current recommendations for the treatment of gonorrhea need to be reviewed and that an on-going surveillance is highly warranted. In view of treatment failures with extended spectrum cephalosporins and detection of isolates resistant or with reduced susceptibility to this class of antibiotics, health agencies from various countries now are recommending ceftriaxone and azithromycin as dual therapy for treatment of gonorrhea (CDC 2012; Bignell & Fitzgerald 2011; HPA 2011; PHAC 2011c). The trend of increased MICs for the extended spectrum cephalosporins in Canada and elsewhere is a critical public health concern. Therefore, an active surveillance i.e. incentive based surveillance is required to monitor emerging trends of cefixime and ceftriaxone reduced susceptibility or resistance in SK where *N. gonorrhoeae* susceptibility to this class of antibiotics is still very high.

4.1.2 High Susceptibility to Azithromycin

High susceptibility of *N. gonorrhoeae* (99.4%) isolates from SK to azithromycin was observed. Azithromycin along with ceftriaxone as a combination therapy has been recommended for the treatment of uncomplicated gonorrhea (CDC 2012; Bignell and Fitzgerald 2011; HPA 2011; PHAC 2011c). In Canada, 0.17% of *N. gonorrhoeae* isolates tested for AMR between 2000 and 2009 were azithromycin resistant (MIC range 2-64 mg/L); however the modal MIC of azithromycin shifted from 0.25 mg/L in 2001 to 0.5 mg/L to 2009 in Canada (Martin et al 2011).

Azithromycin resistant isolates have been detected in USA and the proportion of GISP isolates resistant to azithromycin has remained under 0.5% (CDC 2011; Wu et al 2011). In 2010, 82.4% of gonococcal isolates with azithromycin MICs 8-16 mg/L were from MSM (CDC 2011).

Recently, *N. gonorrhoeae* isolates with high (MIC>512 mg/L) level of azithromycin resistance (Katz et al 2012) and treatment failure with 2 g of azithromycin has been reported from the USA (Soge et al 2012).

In the Western Pacific region during 2010, no azithromycin resistance was recorded from Cambodia, Vietnam and India and very low rates (< 1%) of azithromycin resistance were reported from Australia. By contrast, 34% of tested *N. gonorrhoeae* isolates from Mongolia were resistant to azithromycin (WHO-WPR 2012). *N. gonorrhoeae* isolates with azithromycin MIC > 16mg/L have been detected in Australia (Bates et al 2011).

In Japan, no significant changes were observed in gonococcal susceptibility to azithromycin from 1993 to 2008 (Tanaka et al 2004, 2011). In Hong Kong SAR, 8% (39/485) *N. gonorrhoeae* isolates between 2005 and 2010 were classified as being resistant to azithromycin with an MIC range of 1-256 mg/L (Lo et al 2012). 5.3% (17/485) azithromycin resistance was observed in gonococcal isolates from Nanjing and Chongqing, China (Yuan et al 2011). Azithromycin resistance ranged between 8.2% (5/60) and 38% (20/53) in isolated collected from 2002 through 2007 in Israel (Dan et al 2010).

European Gonococcal Antimicrobial Surveillance Programme of the European Surveillance of Sexually Transmitted Infections (Euro-GASP of ESSTI) reported an increase in azithromycin resistance from 2% (24/1285) in 2008 to 13% (180/1366) in 2009 (Cole et al 2010, 2011). Gonococcal isolates with high (MIC≥256mg/L) level of azithromycin resistance have been detected in Scotland (Palmer et al 2008), Italy (Starnino et al 2009), England and Wales (Chisholm et al 2010) and various parts of Europe (Cole et al 2010).

In Africa, no azithromycin resistance has been detected in Malawi and Kenya in Africa (Brown et al 2010; Mehta et al, 2011).

N. gonorrhoeae isolates with reduced susceptibility (azithromycin MIC=0.25-0.5 mg/L) and resistance (azithromycin MIC⩾1mg/L) had been detected in Latin America and Caribbean countries during the 1990s (Dillon et al 2001a, b, 2006; Sosa et al 2003). Overall, aggregated

azithromycin resistance from 2000 through 2009 in 6 Latin American countries was 13% (1088/8373), ranging from 6% (39/646) of gonococcal isolates tested in 2000 to 23% (225/962) in 2009. Percentage of azithromycin resistant isolates in Argentina decreased from 10% in 2000 to 3% in 2009. Chile reported 3% azithromycin resistance in gonococci tested in 2000 and increased to 46% in 2009 (Starnino et al 2012).

4.1.3 Low Prevalence of Quinolone Resistant *N. gonorrhoeae*

Development of high level of resistance to quinolones in *N. gonorrhoeae* isolates from Canada resulted in the replacement of quinolones as the recommended treatment for gonorrhea in 2007 (Plitt et al 2009; PHAC 2008b, 2010b) by oral cefixime (Mann et al 2003; Sarwal et al 2003; Ota et al 2009; Plitt et al 2009; PHAC 2010b).

Aggregate ciprofloxacin resistance in gonococcal isolates from SK over the nine year period tested in this study (2003-2011) was 4.4% (MIC 0.001-32 mg/L). The annual percentage of ciprofloxacin resistance in SK remained below 5% till 2009 and increased in 2010 (26%) and 2011 (14%). Interestingly, 78% (n=7/9) of ciprofloxacin resistant isolates in 2010 were recovered from Regina and 80% (n=4/5) of ciprofloxacin resistant isolates in 2011 were from Saskatoon. These findings may indicate the presence of an outbreak of ciprofloxacin resistant *N. gonorrhoeae* isolates in the province. Since *N. gonorrhoeae* isolates had remained susceptible to ciprofloxacin for a long period (2003-2009), periodic surveys should be conducted to assess the ciprofloxacin susceptibility of *N. gonorrhoeae* in SK. This finding is interesting because PHAC still recommends quinolones (ciprofloxacin 500 mg or ofloaxcin 400 mg as single dose orally) as an alternative drug for the treatment of gonorrhea (PHAC 2011c).

In Canada, the proportion of gonococcal isolates resistant to ciprofloxacin increased from 1.3% in 2000 to 25.5% in 2009 with a peak of 30.2% in 2007 (Martin et al 2011). Similar trends of gonococcal ciprofloxacin resistance have been reported from Alberta (1.5% in 2001 to 27.7% in 2007; Plitt et al 2009) and Ontario (4% in 2002 to 27.8% in 2006; Ota et al 2009). In a recent study, 29% ciprofloxacin resistance was reported from Ontario, Canada (Allen et al 2011).

The proportion of GISP *N. gonorrhoeae* isolates identified as QRNG in the USA peaked in 2007 at 14.8% (CDC 2009). In the USA, the prevalence of ciprofloxacin resistance in 2010 was 12.5% (CDC 2011).

In many counties from the Western Pacific Region, rates of QRNG are in excess of 90% (WHO-WPR 2012). High prevalences of QRNG have been reported from China (98.7%, Yang et al 2006), Japan (70.7%, Tanaka et al 2011), Korea (86%, Lee et al 2011), India (78%-98%, Khaki et al 2007; Bala et al 2008; Sethi et al 2006) and Israel (27.1%, Dan et al 2010). Prevalence of ciprofloxacin resistant gonococcal isolates continued to increase despite the withdrawal of ciprofloxacin from treatment guidelines (Yang et al 2006). The proportion of isolates with ciprofloxacin resistance in the Western Pacific countries/regions in 2010 ranged between 29.2% (New Zealand) and 100% (India & Vietnam) (WHO-WPR 2012). A high prevalence rate of ciprofloxacin resistant *gonorrhoeae* isolates has been reported from various parts of Europe (Florindo et al 2010; Cole et al 2011; Glazkova et al 2011; Carannante et al 2012); ciprofloxacin resistance in Europe in 2009 was 63% and increased by 12% between 2008 and 2009 (Cole et al 2011). The burden of ciprofloxacin resistance remains disproportionately high in MSM worldwide (HPA 2009; Ota et al 2009; CDC 2009, 2011).

In Africa, most studies on isolates collected before 2000 showed that *N. gonorrhoeae* remained susceptible to the flouroquinolones (Dan 2004; WHO 2001). However, recent studies have shown that QRNG have emerged and spread in various parts of Africa. For example, the prevalence of ciprofloxacin resistance was 7% in Cape Town in 2004 and 11% in Johannesburg, whereas in 2007, percentages of resistant isolates were 27% in Cape Town and 32% in Johannesburg (Lewis et al 2008), respectively. In a recent study, a high prevalence of fluoroquinolone resistance (53.2%) was recorded in *N. gonorrhoeae* isolates from Kenya (Mehta et al 2012). A high level of gonococcal ciprofloxacin susceptibility had been reported from Mozambique (100%) and Malawi (99%) (Apalata et al 2009; Brown et al 2010).

Gonococcal quinolone resistance in Latin America and Caribbean countries was rare in the 1990s although low level resistance was noted in several regions previously (Dillon et al 2006; Dillon et al 2001b; Ison et al 1998; Swanston et al 1997), which reflected the limited use of these drugs in the region (Dillon & Pagotto 1999; Dillon et al 2001a, b). However, aggregate

ciprofloxacin resistance in 11 reporting countries from Latin America increased from 2% in 2000 to 31% in 2009. Significant increases ingonococcal ciprofloxacin resistance has been reported from Argentina (1% in 2000 to 23% in 2009), Chile (1% in 2004 to 46% in 2009) and Peru (8% in 2000 and 60% in 2006) during the last decade (Starnino et al 2012).

4.1.4 Sporadic Prevalence of *N. gonorrhoeae* Resistant to Penicillin

It is interesting to note that penicillin resistance levels in *N. gonorrhoeae* isolates from SK are much lower compared to national Canadian penicillin resistance rates (Martin et al 2011). The overall burden (2003-20011) of *N. gonorrhoeae* resistance to penicillin (MIC $\geq$ 2.0 mg/L) was 4% in SK. It seems that prevalence of penicillin resistance has not changed in SK over the past two decades since in a national study conducted in 1988-89, low (0-0.9%) penicillin prevalence was recorded from SK (Dillon 1989). This can be attributed to the removal of the penicillin from treatment guideline of the gonorrhea in mid 1980s (PHAC 2006) and limited introduction of penicillin resistant isolates from outside the province.

In Canada, the nationwide prevalence of penicillin resistance in *N. gonorrhoeae* isolates decreased between 2000 and 2003 from 14.2% to 5.6% and then increased to 18.7% by 2009 (Martin et al 2011). In a recent study from Ontario, Canada, 12.2 % penicillin resistance was reported (Allen et al 2011).

In the USA, GISP reported approximately 10% penicillin resistance in *N. gonorrhoeae* isolates for each year since the late 80s (CDC 2009).

The proportion of gonococci with penicillin resistance in Western Pacific countries/regions in 2010 ranged between 18.1% (New Zealand) and 96.7% (Thailand) (WHO-WPR 2012). High prevalences of penicillin resistant gonococcal isolates have been reported fromChina (93.1%, Yang et al 2006), Japan (16.1%, Tanaka et al 2011) and Korea (74% to 91%, Lee et al 2011).

The Euro-GASP of ESSTI demonstrated an overall burden of 21% penicillin resistance during 2006 and 2008 (Cole et al 2010). In Portugal, penicillin resistance during 2006 and 2009 was 79.1% (Florindo et al 2010).

In Latin America and the Caribbean in the 1990s, percentages of penicillin resistant gonococcal isolates (MIC ≥ 2.0 mg/L) varied between 48.5% in 1993 and 25.3% of isolates tested in 1999 (Dillon et al 2006). Overall, aggregate resistance percentages to penicillin in 11 countries of Latin America during 2000 and 2009 decreased from 35% to 26%. In 2009, the prevalence of penicillin resistance in Latin America ranged from 16% in Uruguay to 87% of isolates tested in Venezuela (Starnino et al 2012).

The emergence of high-level, plasmid-mediated, penicillinase-producing resistance to penicillin was first reported in 1976 (Percival et al 1976; CDC 1976; Dillon et al 1978b). The overall prevalence of PPNG in SK was 0.9%; 24% of total penicillin resistance in SK. By comparison the number of PPNG strains isolated in Canada 1976-1984 comprised < 1% of all gonococcal isolates (Dillon et al 1986), it increased to 5% in 1985 and was 9.6% in 1990 (Yeung et al 1990, 1991). PPNG isolates carrying the Asia type (80.2%), Africa type (15.8%) and Toronto type (4%) β-lactamase plasmids have been reported from Canada (Dillon et al 1986). The Asia, Africa and Toronto types of β-lactamase producing plasmids have been associated with epidemic outbreaks of gonorrhea (Dillon & Yeung 1989). The prevalence PPNG isolates in Canada decreased from 3.0% in 2000 to 0.9% in 2009. During this period, Africa type plasmids (78.8%) were the most common type among PPNG in Canada followed by the Toronto type (13.8%) and Asia type (6.1%) (Martin et al 2011). A decline in prevalence of PPNG was recorded in the USA from an annual peak of 11.0% in 1991 to 0.4% in 2007 (CDC 2009).

The proportions of PPNG in Western Pacific countries/regions in 2010 ranged from 5.9% in Malaysia to 96.6% in China (WHO-WPR 2012). A PPNG prevalence of 37.8% (2004-2005) and 21% (2006) has been reported from China and Korea (Yang et al 2006; Lee et al 2011). 34.1% of the tested *N. gonorrhoeae* isolates in Bangladesh were PPNG and all of these were African type (Alam et al 2012).

The overall prevalence of PPNG in Europe for 2009 was 12% (Cole et al 2011). The proportions of PPNG isolates in Portugal (2004-2009) and Italy (2006-2010) were 15.5% and 6% (Florindo et al 2010; Carannante et al 2012). Plasmid-mediated penicillin resistance was 65% in Mozambique (2005) and Kenya (2002-2009) (Apalata et al 2009; Mehta et al 2011). 97% of the PPNG detected in Kenya were Africa type and 3% were of Asia type (Mehta et al 2011). 25.8% of the tested *N. gonorrhoeae* isolates in South Africa were PPNG carrying Africa-type (35.2%), the Toronto-type (44.4%), and a new, Johannesburg type (20.3%) plasmid, related to the Asia-type (Fayemiwo et al 2011).

High percentages of PPNG had been noted in Latin America and Caribbean countries during 1990s, ranging from 17.9% to 38.8% (Moreno et al 1987; Dillon et al 2006). A recent survey, in the 7 countries from Latin America, reported an overall PPNG prevalence of 21% (Starnino et al 2012). A significant decrease in PPNG isolates was observed from 31% in 2000 to 20% in 2009 in this region (Starnino et al 2012).

4.1.5 High Prevalence of Tetracycline Resistant *N. gonorrhoeae*

The total burden of tetracycline resistance (MIC $\geq$ 2.0 mg/L) in SK was 51% (2003-2011). High levels of tetracycline resistance in SK can be attributed to the selective pressure exerted through the doxycycline which remains one of the recommended drugs to treat *C. trachomatis* infections (Dillon and Pagotto 1999; PHAC 2010b). By comparison, in a 1988-89 study, tetracycline resistance in SK ranged between 0 to 2.7% (Dillon 1989). Interestingly enough, no resistance to tetracycline was observed in 2009 and 2010 and tetracycline resistance levels were around 5% (2/37) in 2011.

High-level, plasmid-mediated resistance to tetracycline (TRNG, tetracycline MIC $\geq$16 mg/L) was first recorded in mid-1980s (CDC 1985; Roberts et al 1988). The first TRNG isolated in Canada was reported in 1986 (Shaw et al 1986). In this study, TRNG constituted 3.3% of total tetracycline resistance and all the TRNG were Dutch type. The prevalence of tetracycline resistant isolates increased from 14.3% in 2003 to 24.7% in 2009 in Canada whereas proportion of TRNG in Canada has decreased from 3.4% in 2000 to 1.6% in 2009 (Martin et al 2011).

Between 1986 and 1997, 29.1% of *N. gonorrhoeae* isolates tested for antimicrobial susceptibility in Canadian laboratories were TRNG with predominance of Dutch (79.3%) type *tetM* plasmids over the American (20.7%) type *tetM* plasmids (Greco et al 2003).

In the USA, GISP reported that approximately 10% of *N. gonorrhoeae* isolates were resistant to tetracycline since the late 80s (CDC 2007). The prevalence of TRNG in USA was 5.6% in 2007 (CDC 2009).

In Shanghai, tetracycline resistance between 2004 and 2005 was 56.5% which included 6.3% TRNG (Liao et al 2006). The proportion of TRNG has remained high in the countries of the Western Pacific region for many years with range a of 10% to 100% (WHO-WPR 2012). The rates of tetracycline resistant *N. gonorrhoeae* isolates in Japan (2008) and Korea (2000-2006) were 28.1% and 97% (Tanaka et al 2011, Lee et al 2011). TRNG prevalence in Korea from 2000 through 2006 increased from 3% to 9% (Lee et al 2011).

Euro-GASP of ESSTI reported tetracycline resistance of 60% during 2006 and 2008 and TRNG prevalence fairly stable at 16% (Cole et al 2010). Tetracycline resistant isolates (2006-2010) in Italy were 38% (Carannante et al 2012).

Studies in Latin America and the Caribbean in the 1990s reported that the total burden of tetracycline resistance in the region ranged from 28.6% (1994) to 52.5% (2003) (Dillon et al 2006; Dillon et al 2001b; Sosa et al 2003). In a recent 2009 survey, in Latin American the prevalence of tetracycline resistant gonococcal isolates was 35% compared to 60% in 2000 (Starnino et al 2012). Plasmid-mediated tetracycline resistance was 97% in Kenya (Mehta et al 2011) and 77% in Mozambique (Apalata et al 2009).

All the TRNG observed in this study were Dutch type. A higher prevalence of Dutch type plasmids in TRNG than the American type has been reported in Canada, China and Bangladesh (Greco et al 2003; Yang et al 2006; Alam et al 2012). In South Africa, the America type was 3-fold more frequent as compared with the Dutch type (Fayemiwo et al 2011). The overall percentage of TRNG (2000-2009) in five Latin American countries was 10% (Starnino et

al 2012). No isolate with combined plasmid-mediated resistance of PPNG and TRNG (PP/TRNG) resistance was identified in this study. PP/TRNG is common in Canada (Carballo et al 1990; Dillon and Carballo 1990; Martin et al 2011). 455 PP/TRNG strains were detected in Canada between 2000 and 2009 (Martin et al 2011). PP/TRNG strains have been reported from various parts of the world (Yang et al 2006; Bala et al 2008 HPA 2008). The prevalence of PP/TRNG in the USA has continued to be low (0.5%) since 1999 to 2007 (CDC 2009).

4.1.6 Absence of Spectinomycin Resistance in *N. gonorrhoeae* in Saskatchewan

Spectinomycin can be an alternative regimen for treating urogenital gonorrhea in patients who do not tolerate cephalosporins. However, spectinomycin has limited effectiveness for the treatment of pharyngeal infections (CDC 2006). High levels of resistance developed when this antimicrobial was widely used in South-East Asia such as Korea in the mid-1980s (Boslego et al 1987).

We did not observe spectinomycin resistant isolates (MICs ≥ 128.0 mg/L; CLSI, 2009) in SK. However, 3.3 % of isolates with intermediate levels of susceptibility (MIC = 64.0 mg/L; CLSI 2009) were observed. In Canada, one PPNG isolate which was also resistant to spectinomycin has been previously reported (Dillon et al 1978b, 1981). Since the 1990s, spectinomycin resistant gonococcal isolates have not been reported in Canada (Martin et al 2011).

All *N. gonorrhoeae* isolates tested in 2010 from USA were susceptible to spectinomycin. GISP has reported in all five spectinomycin resistant isolates from the USA since 1988 (CDC 2011).

In the mid-1980s, clinical treatment failures caused by spectinomycin resistant *N. gonorrhoeae* strains were observed in US military personnel in the Republic of Korea (Boslego et al 1987). Sporadic gonococcal isolates (12 or less) with *in vitro* resistance or decreased susceptibility to spectinomycin have been reported from Mongolia, China, Bhutan and Bangladesh (WHO-WPR 2012; Ahmed et al 2010). Sporadic spectinomycin resistant isolates

were identified in the Venezuela and Colombia during the 1990s (Dillon et al 2006). All *N. gonorrhoeae* isolates from 2000 through 2009, tested for antimicrobial susceptibility to spectinomycin from 5 Latin American countries were susceptible (Starnino et al 2012). No spectinomycin resistant *N. gonorrhoeae* isolates have been identified in Europe according to published reports (Cole et al 2010, 2011; Starnino et al 2008; Florindo et al 2010; Glazkova et al 2011; Carannante et al 2012).

4.1.7 Conclusions

AMR is an ongoing problem in *N. gonorrhoeae* all over the world. Effective antimicrobial treatment along with early detection of infection or cases is the cornerstone for controlling the disease since no vaccine is available against the disease.

This research provides longitudinal (2003 to 20011) information on the antimicrobial susceptibility of *N. gonorrhoeae* isolated in SK, Canada. This study shows a high prevalence *N. gonorrhoeae* susceptible to currently recommended ceftriaxone and azithromycin; alternatively recommended cefixime and ciprofloxacin and traditionally used but no longer recommended penicillin for treating gonococcal infections in Canada and elsewhere. The susceptibility to these antimicrobials was more than 95%, an accepted definition for an antimicrobial to be used for gonococcal treatment. Recent investigations show that *N. gonorrhoeae* penicillin, ciprofloxacin and azithromycin resistance rates and reduced susceptibility to ceftriaxone in the Northern Territory, Australia are below the WHO threshold of 5.0% (Lahra 2012; Goire et al 2011) . These observations relate to continuous monitoring for AMR and formulation of regional treatment policies based on AMR characteristics of *N. gonorrhoeae* population in an area. The presence of high susceptibility of *N. gonorrhoeae* to penicillin and ciprofloxacin in SK underscores the importance of designing regional surveillance programs to monitor trends of *N. gonorrhoeae* AMR so that antimicrobials can be prudently used to ensure high cure rates for this treatable infection.

4.2 Molecular Mechanisms of Antimicrobial Resistance in *Neisseria gonorrhoeae*

Mechanisms of antibiotic resistance in *N. gonorrhoeae* are of two types, i.e. chromosomally mediated (CMR) and plasmid-mediated resistance (Dillon & Pagotto 1999). Multiple resistance determinants may act synergistically to confer resistance to a single or a number of different antibiotics (Tapsall 2001). Plasmid-mediated resistance in *N. gonorrhoeae* is conferred by acquisition of β-lactamase-producing plasmids (PPNG) or by *tetM*-containing plasmids (e.g. TRNG) (Dillon & Pagotto 1999).

4.2.1 PBP2 Pattern IX, PorB Mutations G120K,A121D and a Combination of *mtrR* Mutation in Promoter (A-) and Multimeric Region (H105Y) are Associated with Reduced Susceptibility to Cephalosporins in *N. gonorrhoeae*

146 *N. gonorrhoeae* isolates from SK collected during 2003 and 2008 were investigated to determine molecular mechanisms of reduced susceptibility to cephalosporins. Sequencing and bioinformatic analysis was performed to determine the associations of mutations in PBP2, MtrR and its promoter, PorB and PBP1 with gonococcal susceptibility to cephalosporins.

The transpeptidase domain of gonococcal PBP2 (*penA*) was polymorphic and exhibited 8 mutation patterns, including the "mosaic" PBP2 pattern XXXIV (cefixime MIC=0.125 mg/L; ceftriaxone MIC=0.06 mg/L), which has been reported to be associated with reduced susceptibility to extended spectrum cephalosporins (Pandori et al 2009; Allen et al 2011; Unemo 2011, 2012a,b). The predominant *penA* mutation patterns for all isolates combined (n=146) were patterns IX, I and XXII. The most common *penA* mutation patterns in isolates from Sydney, Australia were patterns XII, X and XIII (Whiley et al 2007a, b). In this study, of the majority (24/28, 85.7%) of isolates with *penA* pattern XII had high susceptibility to ceftriaxone (MIC≤ 0.0008-0.03 mg/L), whereas pattern X (10/11, 91%) and XIII (11/11, 100%) were associated with higher ceftriaxone MICs (0.06-0.12 mg/L) (Whiley et al 2007a). In Korea, pattern XIII was most common and was present exclusively (28/28, 100%) in isolates with ceftriaxone MICs 0.06-0.25mg/L (Lee et al 2010). In this study, no isolate with *penA* pattern XIII was observed. This pattern has been reported to be one of the predominant *penA* patterns in isolates with higher

MICs to ceftriaxone by others (Whiley et al 2007a; Lee et al 2010). The most common PBP2 mutation patterns in isolates from Shanghai, China were V, XXI and XXVII (Liao et al 2011). Isolates carrying *penA* patterns; V (7/10, 70%), XXI (8/11, 72.7%) and XXVII (5/8, 62.5%) were susceptible to ceftriaxone (MIC 0.004-0.016 mg/L) (Liao et al 2011). In Saskatchewan, gonococcal isolates with pattern IX were predominant in isolates with cefixime (16/24, 67%) and ceftriaxone (14/23, 61%) susceptibility group 2 (cefixime and ceftriaxone MIC $\geqslant$0.03 mg/L). Other predominant *penA* patterns observed in this study, I and XXII were almost exclusively present in isolates in cefixime and ceftriaxone susceptibility group 1 [(pattern I: cefixime, 39/41, 95%; ceftriaxone, 39/41, 95%) and (pattern XXII: cefixime 33/35, 93.3%; ceftriaxone 32/35, 91.4%)].

It has been reported that PBP2 mutations G542S, P551S and P551L are associated with increased ceftriaxone MICs (0.016-0.6 mg/L) (Whiley et al 2010). Structural homology modeling of gonococcal PBP2 have shown that P551S along with F504L, decreases the penicillin acylation rates and may also contribute to reduced susceptibility to cephalosporins (Powell et al 2009). Amino acid substitutions P551S and P551L along with F504L were present in PBP2 patterns IX and XII observed in this study. F504L was also observed in patterns without P551S/L; PBP2 patterns (II, V, XIV, XXII and XXXIV) not associated with isolates with higher MICs ($\geq$0.03 mg/L) to extended spectrum cephalosporins. This observation supports the role of P551S/L in imparting reduced susceptibility to cephalosporins to *N. gonorrhoeae* as reported by others (Powell et al 2009; Whiley et al 2010).

Mosaic *penA* pattern XXXIV observed in this study had N512 and G545S substitutions contributing to reduced susceptibility to cephalosporins but A501substitution, vital to raise cephalosporin MICs above break points was absent (Tomberg et al 2010). A501V substitution is present only in *penA* non-mosaic patterns (Ohnishi et al 2011b). The introduction of A501V by transformation has been shown to increase the ceftriaxone and cefixime MICs of recipient *N. gonorrhoeae* to levels above break points (Tomberg et al 2010). This is attributed to the bulkier side chain of amino acid valine which prevents the active site binding of the cephalosporins by interacting with the alkayl side chain, R1 substituent of the cephalosporin (Tomberg et al 2010).

N512Y substitution in PBP2 contributes to decreased susceptibility to extended spectrum cephalosporins and reversion of substitution N512Y to wild type N512 decreases resistance to cephalosporins (Tomberg et al 2010). N512 is relatively distant from the cephalosporin binding active site but is located along with mutations that are known to be important for resistance to penicillin in non-mosaic PBP2 patterns with spontaneous mutations (Powell et al 2009; Tomberg et al 2010). PBP2 mutations I312M, V316T and G545S mutations in PBP2 are also reported to contribute to higher extended spectrum cephalosporin MICs indirectly in the presence of other PBP2 mutations (Tomberg et al 2010). These mutations have been classified as epistatic mutations which are permissive substitutions of no immediate consequence but stabilize specific elements of the protein and allow it to tolerate subsequent function-switching changes (Ortlund et al 2007). The reversion of these mutations back to the wild type; I312, V316 and G545 decreases the cephalosporin MICs (Tomberg et al 2010). *penA* fragments amplified in this study were between AA 340 and 570, therefore presence of substitutions at amino acid positions I312 and V316 was not observed. Other studies have demonstrated the presence of I312M and V316T substitutions along with G545S in pattern XXXIV (Pandori et al 2009; Ohnishi et al 2011b).

PBP2 pattern IX (D345a, F504L, A510V, A516G, P551L), the predominant patterns observed in this study was significantly associated with decreased susceptibility to ceftriaxone (0.03-0.06 mg/L) and cefixime (0.03-0.125 mg/L) (Susceptibility group 2 isolates). Population studies on gonococcal extended spectrum cephalosporins susceptibility have reported presence of *penA* pattern IX with ceftriaxone MIC 003-0.06 (Whiley et al 2007a) and 0.12-0.25 mg/L (Liao et al 2011). Pattern IX has F504L and P551L substitutions decreasing the acylation of active site but does not contain A501V, N512Y, I312M, V316T and G545S substitutions which have been associated with reduced cephalosporin susceptibility (MIC≥0.25 mg/L) (Powell et al 2009; Tomberg et al 2010; Whiley et al 2010). The elevated MICs (0.03-0.06 mg/L) of cefixime and ceftriaxone of isolates carrying pattern IX can be attributed to decreased acylation of active site due to F504L and P551L substitutions.

Analysis of *mtr* repressor (*mtrR*) and its promoter revealed 12 different mutation patterns derived from mutations in different regions of MtrR. Predomiant *mtrR* mutation patterns observed in this study were G45D, A-; G45D and A-; H105Y.

The most commonly reported *mtrR* mutation is a single base pair deletion (A-) located in the inverted repeat that is between the -10 and -35 the *mtrR* promoter (Lucas et al 1997; Zarantonelli et al 1999; Xia et al 2000; Ng et al 2002; Cousin et al 2004; Tanaka et al 2006). Hagman and Shafer (1995) had proposed that the promoter mutation abrogates *mtrR* transcription. *N. gonorrhoeae* strains containing a single-base-pair adenine deletion (A-) in a 13-bp inverted repeat within the *mtrR* promoter show elevated *mtrCDE* transcription (Lucas et al 1997). Due to this deletion *mtrR* RNA polymerase cannot interact with the mtrCDE promoter that partially overlaps with the *mtrR* promoter at the -35 region (Lucas et al 1997). Population studies have shown the presence of A- in a high proportion of *N. gonorrhoeae* isolates with reduced susceptibility to extended spectrum cephalosporins (Lindberg et al 2007; Lee et al 2010; Allen et al 2011).

Twenty three percent of ceftriaxone and cefixime susceptibility group 1 isolates and approximately 4% in susceptibility group 2 *N. gonorrhoeae* isolates contained an "A" deletion in the 13bp inverted repeat sequence of the *mtrR* promoter. Among all the *mtrR* mutations observed in this study, only A-; H105Y was found associated with both ceftriaxone and cefixime susceptibility group 2 *N. gonorrhoeae*. This combination has not been previously reported in isolates with reduced susceptibility to cephalosporins. The H105Y mutation probably inhibits MtrR dimerization and subsequently further reduce MtrR DNA binding (Shafer et al 1995; Warner et al 2008), or it could enhance binding of antibiotic to MtrR which could then serve as a sink for the antibiotic (Liao et al 2011).

A39T and G45D substitutions abrogate MtrR binding to the target DNA upstream of *mtrCDE* and enhance gonococcal resistance to hydrophobic antimicrobials (Hagman and Shafer 1995; Shafer et al. 1995). In this study, 53% isolates from ceftriaxone and cefixime susceptibility group 1 were with G45D substitution whereas approximately 65% isolates in ceftriaxone and cefixime susceptibility group 2 had this mutation. In this study, no significant association was observed between A39T, G45D or A-/G45D in *mtrR* with any of the cefixime and ceftriaxone susceptibility groups.

PorB, a porin, allows the passage of small molecules, such as β-lactams and tetracyclines, through the outer membrane (Danielsson et al 1986). Mutations at amino acid 120 and/or 121 in PIB alter the conformation of loop3 to decrease the influx of antibiotics into the periplasmic space (Olesky et al 2002). Eleven different mutation patterns in PorB at G120;A121 and one at A121S;N122K were observed in this study. Double mutations in PorB, G120D;A121K have been associated with reduced susceptibility of *N. gonorrhoeae* isolates to extended spectrum cephalosporins (Lindberg et al 2007; Zhao et al 2009; Lee et al 2010, 2011; Martin et al 2012). I also found this to be the case with the isolates studied from SK and gonococcal isolates with G120D;A121K was associated with extended spectrum cephalosporins group 2 isolates. The distribution of PorB mutations observed in gonococcal isolates from SK was different from that reported from Canada in other studies. Population studies on gonococcal AMR from Canada (Allen et al 2011; Martin et al 2012) showed predominance of G120K;A121N in *N. gonorrhoeae* isolates with reduced susceptibility to extended spectrum cephalosporins. This pattern was present in 1% (ceftriaxone) to 2% (cefixime) of cephalosporin susceptibility group 2 isolates.

It has been reported that the L421P mutation in PBP1 is present in *N. gonorrhoeae* isolates which have reduced susceptibility to extended spectrum cephalosporins (Takahata et al 2006; Lindberg et al 2007; Allen et al 2011; Martin et al 2012). But genetic studies had shown that the L421P mutation in PBP1 does not contribute to reduced susceptibility to extended spectrum cephalosporins since PBP1 is not a target for the action of extended spectrum cephalosporins as with penicillin (Zhao et al 2009). A significant association was observed between the L421P mutation in PBP1 and isolates with cefixime and ceftriaxone MICs ≥0.3mg/L.

4.2.2 PBP2 Pattern IX and DNA Binding Domain Mutation G45D in *mtrR* are Associated with Penicillin Resistance in *N. gonorrhoeae*

Not many studies have related PBP2 patterns with penicillin susceptibility. Individual contributions of different PBP2 patterns except for pattern I, X and $penA_{H041}$ (mosaic *penA* allele) to gonococcal penicillin resistance were not determined (Ropp et al, 2002; Zhao et al 2009; Ohnishi et al 2011b). $penA_{H041}$ is present in *N. gonorrhoeae* isolate H041, recently isolated in

Japan with high level of penicillin, ciprofloxacin, cefixime and ceftriaxone resistance (Ohnishi et al 2011b). A significant association was observed between *penA* allele IX and Pen^R (MIC=2-4mg/L) and Pen^I (MIC=1mg/L). This PBP2 pattern has been reported by others to be present in gonococcal isolates with penicillin MICs of 2-128 mg/L (Whiley et al 2007a; Liao et al 2011). PBP2 pattern I was significantly associated with Pen^S gonococci as reported by Whiley and others (2007a). The isolate with mosaic pattern XXXIV was Pen^I (MIC=0.5mg/L). Gonococcal isolates with this pattern have been reported to have Pen^I (MIC=1mg/L) (Allen et al 2011; Unemo et al 2012a) or Pen^R (MIC=2mg/L) (Allen et al 2011).

Analysis of *mtrR* mutations for penicillin resistance revealed the association of *mtrR* mutations G45D and A-;G45D with Pen^I whereas only G45D was significantly linked to Pen^R. This suggests that the substitution G45D plays an important role in the development of gonococcal penicillin resistance. *mtrR* mutation combination A-;H105Y which was significantly associated with cefixime and ceftriaxone susceptibility group 2 had no association with Pen^R.

A high proportion (31%) of the isolates with intermediate susceptibility to penicillin carried G120D and A121N mutations whereas the majority of Pen^R (60%) isolates carried G120K and A121D mutations. This is in agreement with earlier transformation studies which demonstrated that PIB at positions G120 and/or A121 with charged amino acids such as Lys (K), Arg (R), His (H) and Asp (D) are capable of conferring antibiotic resistance in *N. gonorrhoeae*. Substitution with Asp at either position 120 or 121 conferred only partial resistance to penicillin (Olesky et al 2002).

The L421P mutation of PBP1 is also present in *N. gonorrhoeae* isolates resistant to penicillin (Shigemura et al 2005; Lee et al 2010). PBP1 is one of the targets for penicillin action although compared to PBP2, a 10-fold higher concentration of penicillin is required to act on PBP1 to inhibit penicillin susceptible gonococcal strains (Barbour, 1981). A significant association was observed between Pen^I and Pen^R and the L421P mutation in PBP1as did Lee et al (2010) and Ropp et al (2002).

4.2.3 Association of GyrA, ParC and MtrR Mutations with Ciprofloxacin Resistance in *N. gonorrhoeae*

The combinations of the amino acid substitutions in GyrA and ParC of gonococcal QRDRs form diverse mutation patterns (Uehara et al 2011; Starnino et al 2010; Chen et al 2010; Yang et al 2006; Giles et al 2004; Su& Lind 2001; Yoo et al 2004; Ng et al 2002b; Trees et al 1999, 2001). Four QRDR mutation patterns were observed in ciprofloxacin resistant (MIC≥ 1mg/L) and intermediately susceptible (MIC≥ 0.5mg/L) isolates in this research. The majority of the reported QRDR mutation patterns have a double mutation in GyrA (Ser91Phe and Asp95Gly/Asn), combined with a single mutation (Ser-87Arg) in ParC (Starnino et al 2010; Chen et al 2010; Ng et al, 2002). In this study, mutations in gonococcal ParC (Ser87Arg and Ser87Arg and Ser88Pro) were present in 2 isolates with intermediate susceptibility (MIC=0.5 mg/L) to ciprofloxacin. This is contrary to some studies in which ParC mutations have been correlated with high level of quinolone resistance (Belland et al 1994; Tanaka et al 1998).

Bacterial efflux pumps that export antibiotics can have important implications for the efficacy of antibiotic treatment of infections (Nikaido 1994). Mutation in genes encoding drug efflux pumps also contribute to resistance along with mutations in enzymes GyrA and ParC (Ilina et al 2008; Marcusson et al 2009). In this study, ciprofloxacin resistance was also significantly associated with *mtrR* mutation, A-;H105Y and both the isolates with ciprofloxacin intermediate susceptibility were linked with truncated *mtrR*. These *mtrR* alterations, A-;H105Y and truncated *mtrR* have not been reported in earlier studies investigating the role of *mtrR* mutation in imparting ciprofloxacin resistance to gonococci (Starnino et al 2010; Chen et al 2010).

It has been suggested that high percentages of ciprofloxacin resistant isolates can be maintained in a population because of the fitness advantage provided by mutations in GyrA (Kunz et al 2012; Dillon & Parti 2012, Sadiq et al 2010). But this advantage can be abrogated by a mutation in *parC,* which confers a high level of resistance and fitness can be restored by compensatory mutations in *mtrR* and GyrA. The survival fitness of ciprofloxacin resistant gonococci in Saskatoon since the gonococcal isolates after 2008 were not available for DNA sequence analysis. Ciprofloxacin resistant isolates detected in our study had mutation in GyrA,

ParC and *mtrR* but at residues different from those investigated in survival fitness (Kunz et al 2012; Dillon & Parti 2012). QRNG may last for years even though selection pressure has been removed by dropping ciprofloxacin from treatment guidelines for gonorrhea (Kunz et al 2012; Dillon & Parti 2012). Therefore, continued surveillance of QRNG is required in SK where QRNG are emerging much later compared to other parts of the world.

4.2.4 Role of *mtrR* and 23SrRNA Mutations in Azithromycin Resistance in *N. gonorrhoeae*

Azithromycin belongs to the macrolide-lincosamide-streptogramin family and can be administered orally in a single dose to treat gonorrhea (WHO 2003). Multiple mechanisms of resistance to azithromycin have been recorded. These involve A-, TT+ (dinucleotide insertion in *mtrR* promoter), A39T, R44H and A-;H105Y in *mtrR* and C2611T and C2599T in 23S rRNA (Galarza et al 2010; Wu et al 2011; Soge et al 2012). Two azithromycin resistant *N. gonorrhoeae* (MIC=2mg/L & 8mg/L) detected in this study had C2611T mutation in all four alleles of 23S rRNA along with a G45D in *mtrR* DNA binding domain.

In this study, all isolates with azithromycin MIC 1.0 mg/L had *mtrR* mutations; A39T, G45D, A-/G45D and A-/H105Y. We did not find 23S rRNA methylase genes (*ermA*, *ermB*, *ermC* and *ermF*) in any of the gonococcal isolates investigated for molecular mechanisms of azithromycin resistance.

The detection and isolation of strains with resistance to azithromycin is of concern especially in the context of the emergence of cephalosporin resistance among *N. gonorrhoeae* and recommendation of azithromycin along with ceftriaxone for treatment of gonococcal infections. Emergence and spread of strains of *N. gonorrhoeae* with azithromycin resistance will further limit the treatment options available for *N. gonorrhoeae* infections.

High-level azithromycin resistance (≥256 mg/L) develops rapidly with a single mutated allele of 23S rRNA. It presents the possibility that high-level azithromycin resistance could develop from an apparently sensitive isolate *in vivo* under a selection pressure. This can occur easily in gonorrhea patients prescribed azithromycin to treat *C. trachomatis* co-infection.

(Chisholm et al 2010). The development of *N. gonorrhoeae* strains with high level of azithromycin resistance by a point mutation in the 23S rRNA highlights the importance of treating gonorrhea with recommended therapies to prevent selection and dissemination of gonococcal strains with high levels of azithromycin resistance.

4.2.5 Specific Combinations of Individual Mutations *penA*, *mtrR* and *porB* are Associated with Gonococcal Cephalosporin and Penicillin Susceptibility

The combined mutation patterns in *penA*, *mtrR* and *porB* were associated with gonococcal susceptibility to extended spectrum cephalosporins and penicillin. Ilina and others (2008) demonstrated the presence of *penA*, *porB*, *ponA*, *mtrR*, *gyrA* and *parC* mutations implicated in gonococcal AMR in *N. gonorrhoeae* isolates susceptible to penicillin, tetracycline and ciprofloxacin. *penA/mtrR/porB* combined mutation pattern I/WT/WT and was significantly associated with *N. gonorrhoeae* isolates susceptible to extended spectrum cephalosporins and Pen^S. In this study, isolates with higher cefixime MICs (0.03-0.125; Group 2) were found to be significantly associated with pattern IX/ G45D/ G120K;A121D. Penicillin resistance was significantly associated with combined mutation patterns, IX/ G45D/G120K;A121D and IX/G45D/G120D;A121D.

These findings indicate that certain combined mutation patterns may be predictive of antimicrobial susceptibility. Increasing use of NAATs for the diagnosis of gonorrhea on clinical specimens (urine) had resulted in lesser recoveries of *N. gonorrhoeae* clinical isolates. Thus MIC determination on clinical isolates has become impossible in most cases. Therefore, molecular methods of diagnosis based on combined mutation patterns can detect antimicrobial resistance and predict trends and emergence of AMR from non invasive specimens.

4.2.6 Conclusions

This research systemically examined mutations in various loci in association with gonococcal resistance to different antibiotics. It is the first report with regards to molecular mechanisms of resistance to various antibiotics including extended spectrum cephalosporins in

N. gonorrhoeae isolates from Saskatchewan. Results of this study show that the molecular factors associated with high MICs of cefixime and ceftriaxone include a PBP2 pattern IX, simultaneous mutations (A-; H105) in MtrR promoter and multimeric region, and PorB substitution at G120 and A121 with charged amino acids. The correlation of A-/H105 in higher extended spectrum cephalosporins (cefixime and ceftriaxone) was demonstrated for the first time in this study. Penicillin resistance was correlated to G45D substitution in MtrR DNA binding domain mutations and combination of adenine deletion in *mtrR* promoter and G45D (A-; G45D). This study is the first to identify the occurrence of multiple mutations in azithromycin resistant isolates from Canada. Investigation of molecular determinants of resistance i.e. PBP2, MtrR, and PorB revealed that specific combination of mutations involving individual alterations in these genes are associated with different cefixime and penicillin susceptibility and resistance phenotypes.

4.3 Population Dynamics and Evolution of Antimicrobial Resistance in *Neisseria gonorrhoeae* in Saskatchewan

This study confirms that both *porB* DNA sequence analysis and NG-MAST analysis have high discriminatory powers sufficient to distinguish *N. gonorrhoeae* isolates and to identify circulating clusters of strains. The indices of discrimination recorded in this study for modified (765bp; ≈77% of *porB* sequence) *porB* typing and NG-MAST were 0.94 and 0.96. These findings are in line with the recent studies comparing these two typing schemes (Liao et al 2009; Ilina et al 2010; Heymans et al 2011).

NG-MAST appears to be a leading method for *N. gonorrhoeae* typing (Martin et al 2004; Unemo & Dillon 2011) with an index of discrimination (ID) ≥95% (Liao et al 2009; Ilina et al 2010; Heymans et al 2012; Kushnir et al 2012).

It has been suggested that *porB* typing is less expensive and more rapid than sequencing the entire *porB* gene and NG-MAST and provides comparable ID (Liao 2009). The modified *porB* typing requires single PCR reaction and 2 DNA sequencing reactions. To obtain sequences of the entire *porB* gene, four DNA sequencing reactions for each strand are often needed to

achieve unambiguous sequence results (Liao et al 2009). NG-MAST typing also involves two PCR and four DNA sequencing reactions.

The index of discrimination of MLST scheme based on seven housekeeping genes used in this study for *N. gonorrhoeae* isolates (2005-2009) was 0.89. Ilina et al (2010) had reported ID value of 0.91 by MLST with seven housekeeping genes. The discriminatory ability of the present MLST schemes, examining seven housekeeping loci, is suboptimal (<0.9). Therefore, it has been suggested that to differentiate clonal populations detected by MLST, the loci sequenced should also include more rapidly evolving loci in addition to housekeeping genes (Urwin & Maiden 2003). A higher ID value of 0.953 has been reported for MLST using 10 housekeeping loci (Vidovic et al 2011).

porB typing and NG-MAST have high ID but result in over discrimination of *N. gonorrhoeae*. In my study, this observation was highlighted from the fact that 3 major MLST-STs (ST-1, ST-2 and ST-3) comprising 101 (2005-2008) isolates were differentiated into 26 STs each by *porB* typing and NG-MAST. MLST ST-1, ST-2 were ST-3 resolved into 15 and 16 STs, 9 and 7 STs and 6 and 4 STs by *porB* typing and NG-MAST. This has also been observed by others for *porB* typing and NG-MAST (Ilina et al 2010; Mavroidi et al 2011). It has been suggested that distorting effects among genetically related isolates can arise in typing schemes that are based on the hyper-variable genes. For example, *porB* and *tbpB* DNA sequence can be subjected to frequent mutation and recombination, this may lead to a failure to identify the predominant related strains of *N. gonorrhoeae* that circulate within community (Vidovic et al 2011). MLST which is based on concatenated sequences of house- keeping has the ability to provide a buffer against the distorting effects of mutations and recombinations resulting in the maintenance of clonal stability of *N. gonorrhoeae* isolates.

4.3.1 Clustered Distribution of Antimicrobial Resistance/Susceptibility in *N. gonorrhoeae* Revealed by *porB*-based Typing, NGMAST and MLST Analysis

All the three typing schemes i.e. modified *porB* typing, NG-MAST and MLST used in this study revealed that antibiotic resistant and susceptible *N. gonorrhoeae* isolates in SK belong

to different clusters. Three predominant STs observed through *porB t*yping and NG-MAST accounted for 36% and 28% of 320 gonococcal isolates typed with these schemes. Both for *porB* typing and NG-MAST more that 50% of isolates were grouped under ≤10 STs.

The average number of isolates per NG-MAST ST was 3.9 and the number of isolates per *porB* ST was 4.5. The average number of isolates per ST observed through *porB* typing and NG-MAST in this study was higher compared to those reported by others (Unemo et al 2007; Olsen et al 2008; Ilina et al 2010). The reported number of isolates per *porB*-ST ranged from 1.24 (Unemo et al 2003) to about 2.0 isolates (Unemo et al 2007; Olsen et al 2008; Liao et al 2009; Ilina et al 2010) and for NG-MAST the range was 1.7 (Unemo et al 2007; Olsen et al 2008; Ilina et al 2010) to 2.4 isolates per ST (Martin et al 2004; Palmer & Young 2006; Abu-Rajab et al 2009; Liao et al 2009). Large gonococcal clusters were identified by the *porB*-based typing and NG-MAST. For example, 57 isolates exhibited an identical *porB* ST 109, and 46 of these isolates also displayed a single NG-MAST ST 25. Identification of a large group of isolates as single ST and high average number of isolates per *porB* and NG-MAST ST implies that *N. gonorrhoeae* isolates in SK are related by genotype.

Molecular epidemiological studies can provide novel information on the emergence and dissemination of antimicrobial resistant gonococcal clones. Tetracycline resistance was present across the majority of STs detected through *porB* typing and NG-MAST in this study. TRNG (tetracycline MIC ≥16 mg/L and β-lactamase negative) had a clustered distribution and were exclusively associated with *porB* ST-130 and NG-MAST ST-3711. The endemic presence of tetracycline resistance may be attributed to the history of tetracycline and doxycycline use in the co-treatment of chlamydial infections as these antibiotics are not recommended for the primary treatment of gonorrhoea in Canada (PHAC 2010b). CMRNG (69%) observed in this study appeared as *porB* ST-111 and NG-MAST ST-3654.

All the isolates resistant to ciprofloxacin (n=5) and azithromycin (n=2) appeared as single *porB* and NG-MAST STs suggesting a sporadic presence of AMR to these antimicrobials. Around 40 % of the isolates susceptible to all the tested antimicrobials in this study were grouped as *porB* ST-109 and NG-MAST ST-25.

Several studies have shown a clustered distribution, as ascertained by NG-MAST analysis, of *N. gonorrhoeae* isolates which are resistant to ciprofloxacin, azithromycin orwith reduced susceptibility to extended spectrum cephalosporins (Palmer et al, 2005; Palmer & Young 2006; Moodley et al 2006; Palmer et al 2008; Starnino et al 2008, 2009; Monfort et al 2009; Martin et al 2012; Heymans et al 2012; Buono et al 2012) and *porB* typing (Liao et al 2008) or both (Lundback et al, 2006; Unemo et al 2007; Lindberg et al 2007; Ilina et al 2010). Not many studies have shown clusters of *N. gonorrhoeae* isolates resistant to penicillin and tetracycline as determined using NG-MAST or other molecular typing methods.

The grouping of predominant *porB* and NG-MAST STs and on the basis of <1% bp difference from predominant STs indicated that the gonococal population in SK is related. The grouping of predominant *porB* and related *porB* STs (STs with 1-7 bp difference from predominant *porB* ST) together stratified 320 isolates into 13 groups with ID of 0.87. *porB* groups 109 and 108 were the most prevalent groups followed by *porB* group 111. *porB* groups 108 and 141 were the major groups in CMTR isolates. *porB* group 109 was the predominant group in susceptible isolates. The classification of the NG-MAST STs (NG-MAST group), sharing one identical allele *(porB* or *tbpB*) and differing by ≤ 1% bp at the other NG-MAST allele identified 29 NG-MAST groups with an ID of 0.93. The major NG-MAST groups were 25, 3655 and 921. In CMTR isolates, 12 NG-MAST groups were observed. NG-MAST group 25 was predominant in susceptible isolates.

One isolate in this study appeared as NG-MAST ST1407, ciprofloxacin MIC 8.0 mg/L, ceftriaxone MIC 0.125 mg/L, cefixime MIC 0.06 mg/L in this study. NG-MAST ST1407 carried PBP2 mosaic pattern XXXIV, was recovered in 2006 from SK, and was first identified in Scotland in 2007. This strain type has been marked as one of the most successfully distributed *N. gonorrhoeae* strain types all over the world and with resistance to penicillin, tetracycline (TRNG), ciprofloxacin and reduced susceptibility to extended spectrum cephalosporins (Florindo et al 2010; Golparian et 2010; Tapsall et al 2010; Eastick 2010; Tanaka et al 2011; Unemo et al 2012; Martin et al 2012; Hess et al 2012; Chisholm et al 2013). ST1407 has been reported from Canada (Martin et al 2012), but only one such isolate was observed in this study involving screening of 320 isolates obtained over a period of 6 years (2003-2008). The prevalence this NG-MAST ST is needed to be determined in the province after 2008, since isolates from 2010 and

2011 had shown a substantial increase in ciprofloxacin resistance commonly associated with ST1407, along with reduced susceptibility to extended spectrum cephalosporins.

The average number of isolates per MLST ST was 6.7 whereas 3.4 and 9.8 *N. gonorrhoeae* isolates per MLST-ST were observed using different MLST schemes from the one used in this study (Ilina et al 2010; Mavroidi et al 2011). Three predominant STs were observed through MLST accounting for 52% of the 193 gonococcal isolates typed. The predominant MLST ST-1 was found as an ancestor of the major clonal complex, suggesting that this circulating strain has been present in SK for a long period. With MLST, 46 *N. gonorrhoeae* isolates were ST-1 and these same isolates exhibited 15 and 16 STs through NG-MAST and *porB* typing. All the TRNG in MLST appeared as MLST ST-20 which is not associated with any other antibiotic resistant and susceptible isolates. In MLST, CMRNG were observed among the major gonococcal lineages, further suggesting that this phenotype was probably acquired in SK as a result of spontaneous mutations in established lineages (ST-1, ST-3 and ST-25). The clonal complexes with SLV observed through MLST and eBURST analysis had two to five STs with the number of gonococci ranging from two to 52 isolates. The depiction of ciprofloxacin resistance across the gonococcal lineages on the minimum spanning tree suggests that ciprofloxacin resistance was acquired independently from genetically distant lineages rather than by clonal expansion. All gonococcal lineages resistant to ciprofloxacin were positioned at the edges of the minimum spanning tree, indicating a very distant genetic relatedness to the major gonococcal lineages and further suggesting that ciprofloxacin resistance was most likely introduced from outside of the province. In MLST, susceptible isolates were grouped under MLST ST-29 and had a SLV from MLST ST-24.

This study for the first time demonstrated clonal expansion of resistance determinants of *N. gonorrhoeae* on the basis of NG-MAST. Specific PBP2/MtrR/PorB mutation combinations were found to be associated with NG-MAST STs. Isolates with NG-MAST ST 25 were associated with PBP2 (*penA*)/MtrR (*mtrR*)/PorB (*porB*) pattern I/WT/WT and with antibiotic susceptibility. NG-MAST ST 3654 was associated with *penA/mtrR/porB* pattern IX/G45D/G120K;A121D and resistance phenotype CMRNG, CMTR isolates and isolates of

cefixime susceptibility group 2. Isolates with chromosomal resistance to tetracycline were significantly ($P<0.05$) associated with several STs and *penA/mtrR/porB* patterns.

This is the first approach where regional AMR data along with molecular typing has been used to determine dissemination of molecular determinants of resistance. This can provide enhanced surveillance of AMR in gonorrhoea since the presence of different STs from a region can be correlated with occurrence of AMR to specific antibiotic(s). Future studies involving higher number of gonococcal isolates recovered from larger populations will help in establishing more reliable and better correlation between occurrence of AMR and STs.

4.3.2 *porB* Typing and NG-MAST is a Useful Tool for Short Term Epidemiological Studies in *N. gonorrhoeae*

Short term epidemiological or microepidemiological analysis focuses on the identification of community epidemics; strains in an entire population over a limited time (6 months to on year); strains from sexual networks; identifying the emergence and transmission of individual (e.g., antimicrobial-resistant) strains; confirmation or discrimination of presumed epidemiological connections in suspected clusters of infection; contact tracing, and the characterization of bacterial clones. It has been suggested that full- or extended length *porB* sequence analysis and NG-MAST which are based on hyper-variable loci are more suitable for microepiemiological studies (Unemo & Dillon 2011). Both *porB* typing and NG-MAST methods can identify clusters of circulating strains and their epidemiological links (Choudhury et al 2006; Liao et al 2009).

The *porB* ST-108, the second most predominant ST observed in this study had a single bp difference from ST 114 and 131. Two bp differences were observed between ST-114 and 131. All these STs were grouped as *porB* group 108 on the basis of <1% bp difference in amplified *porB* sequence. This means that a large cluster of similar isolates i.e *porB* group 108 was divided into 3 different STs; 108, 114 and 131 due to minor alterations in a hyper-variable gene, *porB.*

Major NG-MAST STs detected during this study shared either *porB* (508:3656) or *tbpB* alleles (25:3657; 3654:3653 & 508:3655). *tbpB* alleles of NG-MAST ST-508 (*tbpB* 28) and NG-MAST ST-3656 (*tbpB* 821) had only one bp difference and appeared as CMTR and CMRNG in this study. STs 508 and 3655 NG-MAST STs were predominantly associated with CMTR *N. gonorrhoeae* isolates and had only one bp difference in *porB* (356:2195) and grouped as NG-MAST group 3655.

These finding clearly show that minor variations in hyper-variable genes utilized in *porB* typing NG-MAST prevented assigning new STs to the similar gonococcal strains. This suggests that large clusters of *N. gonorrhoeae* isolates observed over the years have been classified to different strain types. These STs gradually diminish and appear as new STs owing to mutations in *porB* and *tbpB*. This suggests that *porB* typing and NG-MAST is a better scheme for molecular epidemiological studies on *N. gonorrhoeae* isolates.

4.3.3 MLST Analysis is Useful in Determining the Long Term Epidemiological Studies and Evolution of Antimicrobial Resistance in *N. gonorrhoeae*

Macroepidemiology, or long-term or global epidemiology involves the study of bacterial population dynamics over many years or decades. Therefore, a typing scheme used for studying macroepidemiology should be based on several conserved and evolutionarily neutral genes (Unemo & Dillon 2011). Genetic relatedness of a bacterial population is more certain through MLST genotyping, method based on housekeeping genes than genotyping methods based on antigen-encoding genes such as *porB* and *tbpB* in *N. gonorrhoeae*. These antigen-encoding genes are under positive selection pressure and change rapidly over the short term (Urwin & Maiden 2003; Vidovic et al 2011).

MLST appears to be more suitable for long-term epidemiological studies than is NG-MAST and *porB* typing. This conclusison was drawn on analyzing major MLST-STs (STI-1, ST-2, ST-3 and ST-5) present in all the four years (2005-2008), and their comparison with NG-MAST and *porB* STs. Forty-six *N. gonorrhoeae* isolates clustered as ST-1 by MLST were differentiated into 15 and 16 STs through NG-MAST and *porB* typing. MLST ST-2 (n=24) were

resolved as 7 NG-MAST STs and 9 *porB* STs. 31 isolates associated with MLST ST-3 were grouped as 4 NG-MAST STs and 6 *porB* STs. Only one *porB* and NG-MAST ST appeared for all the four years in isolates grouped under MLAST STs; 1, 2 and 3. This shows that *porB* typing and NG-MAST, which are based on hyper-variable genes, have less genetic stability i.e. more genetic variation between similar STs. Therefore, typing schemes such as MLST, which are based on conserved housekeeping genes, are more suitable for macroepidemiological studies, and the typing schemes revealing hyper-variable loci are more useful with short-term studies.

4.3.4 Conclusions

This research used three different typing methods i.e., *porB* typing NG-MAST and MLST and established a clustered distribution of gonococci in SK. The STs identified by typing schemes based on hyper-variable genes, modified *porB* based typing and NG-MAST were further grouped into MLST strain types, a typing scheme based on housekeeping genes. The MLST scheme used in this study was able to demonstrate that the gonococcal population in SK has evolved within the province with very little intrusion from outside. It is suggested that molecular epidemiological studies based on conserved genetic markers along with those based on hyper-variable loci will provide a desired balance of genetic stability and ability to discriminate.

Significant associations were observed between mutation pattern combinations in *penA*, *mtrR* and *porB* and STs. This indicates that certain combined mutation patterns can be predictive of antimicrobial susceptibility, and are useful for molecular diagnosis.

The predominant *porB* and NG-MAST STs observed in this study differed by minor variations in DNA sequences. The predominant STs detected through MLST were present throughout the study period. It is concluded that MLST is a better epidemiological tool for long term studies, whereas NG-MAST and *porB* typing are more suited for short term studies. It is important that standardized guidelines are established for using the *porB* based typing scheme, which has a sufficient index of discrimination and is cheap and less time consuming, compared to NG-MAST and full length *porB* typing. A *porB* based DNA sequence database for *N.*

gonorrhoeae should be established comparing molecular epidemiology of *N. gonorrhoeae* worldwide.

This study shows a clear association between AMR and STs. The molecular epidemiological tools used in this study have demonstrated clustered distribution of AMR among gonococcal isolates from SK. This highlights the potential of molecular epidemiological typing as a tool to predict AMR.

CHAPTER FIVE

CONCLUDING REMARKS

Gonorrhea, caused by the Gram-negative bacterial pathogen *N. gonorrhoeae*, remains a global public health problem. Control of gonorrhea relies on comprehensive public health strategies, including safe sex, early diagnosis combined with effective treatment of the infection, partner notification, and the identification of sexual networks and high-risk populations in order to implement control and effective prevention programs. In the absence of a vaccine, antibiotic treatment is the cornerstone of gonorrhea control programs. The number of different antibiotics that can be used to treat gonococcal disease is increasingly restricted due to the resistance of *N. gonorrhoeae* isolates to most antibiotic classes such as β-lactams and quinolones.

Currently, gonococcal infections are mostly treatable with extended spectrum cephalosporins alone or in combination with azithromycin. However, increasingly numerous reports of *N. gonorrhoeae* strains with reduced susceptibility or resistance to extended spectrum cepalosporins and treatment failures with these agents, especially with oral cephalosporins (e.g. cefixime), have highlighted the urgent need to discover and assess new therapeutic regimens and antimicrobial agents. For this reason, the World Health Organization (WHO) issued a global alert on potentially untreatable gonococcal infections and calling for a variety of measures to combat drug resistance, including increased antimicrobial susceptibility surveillance.

This research provides temporal (2003 to 2011) and spatial information on gonococcal AMR in SK, Canada. It is the first study investigating the antimicrobial susceptibility, mechanisms and molecular epidemiology of AMR, and the population dynamics of *N. gonorrhoeae* isolates in the province. Tetracycline resistance was widespread in the gonococcal population in SK. *N. gonorrhoeae* isolates in SK are highly susceptible (>95%) to extended spectrum cephalosporins and azithromycin, currently the recommended antibiotics for gonorrhea treatment, and to ciprofloxacin and penicillin, antibiotics which are no longer recommended to treat gonorrhea. According to the accepted definition of gonococcal treatment efficacy, a cure rate of over 95% is required for an antibiotic to be recommended for gonorrhea treatment. Thus, an antibiotic should not be used in situations where resistance is observed in greater than 5% of gonococcal isolates tested. The low levels (<5%) of gonococcal resistance to penicillin and ciprofloxacin observed in this research emphasizes the need to develop regional surveillance programs and treatment guidelines for the prudent and possibly extended use of antibiotics. Such

antibiotic susceptibility in *N. gonorrhoeae* as found in SK is rare and has been noted in only a few regions; the Northern Territory, Australia and Western Pacific Islands of Tonga, New Caledonia and Papua New Guinea.

As a result of evolutionary changes, bacteria have developed various ways to counter the actions of antimicrobials through development of resistance to one or multiple drugs. Gonococcal resistance to antimicrobial agents can be plasmid-mediated, chromosomally mediated resistance or both. In gonococci, plasmid-mediated resistance confers high levels of resistance to penicillin (i.e. PPNG) or tetracycline (i.e. TRNG). Mutations at various loci, singularly or in combination, are associated with chromosomally mediated resistance. The accrual of chromosomal mutations can confer resistance to multiple antibiotics or higher levels of resistance to single antibiotics. The potential array of molecular determinants for antimicrobial resistance in *N. gonorrhoeae* include the quinolone resistance determining regions in *gyrA* and *parC* for quniolones and various mutations in *penA, mtrR, porB* and *ponA* for penicillin, tetracycline and extended spectrum cephalosporin. Azithromycin resistance in *N. gonorrhoeae* is the result of alterations in 23S rRNA and *mtrR* and acquisition of *erm* encoding rRNA methaylases.

This research systemically examined mutations in various loci in gonococcal isolates from SK resistant to penicillin, ciprofloxacin, azithromycin and with higher MICs (>0.03 mg/L) to cefixime and ceftriaxone. It is the first report on emerging molecular mechanisms of resistance in *N. gonorrhoeae* isolates from the province. This study revealed that the gonococcal antimicrobial susceptibility/resistance to extended spectrum cephalosporins and penicillin is associated with simultaneous presence of specific mutations in PBP2 (*penA*), PorB and MtrR. This is the first study to establish the association of PBP2 mutation pattern IX (D345a, F504L, A510V, A516G and P551L) and MtrR mutation A-/H105Y (i.e.an adenine deletion (A-) in the *mtrR* promoter and a H105Y susbstituion in dimerization domain of MtrR with gonococcal isolates with higher MICs to cefixime and ceftriaxone).

Gonorrhea transmission occurs through groups of people called core groups and bridging populations, with the individuals in the bridging group acting as a link between the infected core

groups and the general population. Therefore, effective identification of core groups is critical for the successful control of gonorrhea transmission. Molecular epidemiology, combinational application of molecular tools such as DNA sequence based typing and traditional epidemiological studies may be applied for this purpose. DNA sequence based typing (*porB* based typing, NG-MAST and MLST) for *N. gonorrhoeae* isolates can identify isolates with common molecular characteristics, grouping them into STs, and thereby providing insight into presumptively linked cases. The DNA sequence based typing methods have high resolution power, and coupled with phylogenetic analysis they provide comprehensive information on the genetic relatedness of different strains.

Using *porB*-based typing, NG-MAST and MLST, novel patterns of gonococcal strain distributions were revealed. *porB* based typing and NG-MAST are based on hyper-variable loci and classify the large cluster of similar *N. gonorrhoeae* isolates with minor (1 bp) variations under different STs over the period of time. These typing methods have high discriminating ability but less genetic stability, and are better suited for molecular epidemiological studies of *N. gonorrhoeae* isolates collected over short period of time. This research shows that predominant STs in SK differ over time and across geographical locations. The gonococcal population isolated from major locations in SK is genetically and phenotypically different as compared to other Canadian provinces. Further, *porB* groups and NG-MAST groups, (STs with less than ≤1% bp difference), revealed that the gonococcal population in SK is highly related.

MLST is based on internal DNA sequence fragments of conserved house-keeping genes, which are evolutionarily relatively neutral genes. MLST revealed that the gonococcal population over time in SK is related with very little incursion from imported strains. MLST analysis of AMR demonstrated that ciprofloxacin and azithromycin resistances in *N. gonorrhoeae* in SK evolved through convergent acquisition and were distantly related to predominant MLST gonococcal lineages observed in SK. Penicillin and tetracycline resistance in SK were the result of spontaneous mutations in already established lineages. Certain *porB* and NG-MAST STs were clustered under MLST STs. This underscores that typing schemes, such as MLST, which are based on conserved housekeeping genes, are more suitable for macroepidemiology or long term epidemiological studies. Based on three typing methods used in this study, the best approach to

achieve desired balance of genetic stability and discrimination power in molecular epidemiological studies can be using a combination of conserved genetic markers and hyper-variable loci. In many studies, only a single method is used.

Significant associations were observed between different resistance phenotypes and specific mutation pattern combinations in PBP2, MtrR and PorB and specific NG-MAST STs. This indicates that certain STs with specific combined mutation patterns be predictive of antimicrobial susceptibility and is useful for molecular diagnosis.

Based on this research and previous research an international database of gonococcal *porB* sequences is being established. *porB* typing (756 bp) is less expensive compared with other DNA base typing schemes (full length *porB* typing, 987bp; NG-MAST and MLST) available for *N. gonorrhoeae* strain typing and has good (>90%) resolution power. This will help in globally tracking the strain distribution and transmission of *N. gonorrhoeae*.

This research has been communicated to the SDCL, Regina as well as various other authorities in the STI field provincially, nationally and internationally. High levels of antimicrobial susceptibility in *N. gonorrhoeae* will impact formulation of disease control strategies and treatment guidelines/options for gonorrhea. The findings of this study stress the need to develop regional policies on treatment guidelines for *N. gonorrhoeae* infections and gonococcal AMR surveillance programs. The significant associations between antimicrobial resistance phenotypes/genotypes and specific STs observed in this study suggest that such observations can aid in molecular diagnosis of antimicrobial susceptibility in gonococci. This means that the population studies based on molecular typing that did not involve determination of antimicrobial susceptibility and molecular determinants of resistance in gonococcal isolates can still provide information on resistance phenotype of *N. gonorrhoeae.* This finding is relevant in the present context of gonococcal diagnosis when NAATs are used for the confirmation of *N. gonorrhoeae* infections and less use is made of culture identification methods for the determination of antimicrobial susceptibility.

CHAPTER SIX

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APPENDIX-I

Table A.1 Penicillin susceptibility of 427 *N. gonorrhoeae* isolates from Saskatchewan (2003-2011)[a]

Year[b]	n	% Susceptible (n)	% Intermediate (n)	% Resistant (n)	MIC range (mg/L)	MIC_{50} (mg/L)	MIC_{90} (mg/L)
2003	60	13 (8)	80 (48)	7 (4)[c]	0.06-16.0	0.25	1.0
2004	59	31 (18)	63 (37)	7 (4)	0.016-4.0	0.25	1.0
2005	52	12 (6)	76 (40)	12 (6)[c]	0.016-32.0	0.5	2.0
2006	55	20 (11)	80 (44)	0 (0)	0.008-1.0	0.125	1.0
2007	53	17 (9)	83 (44)	0 (0)	0.008-1.0	0.5	1.0
2008	41	17 (7)	81 (33)	2 (1)	0.008-2.0	0.25	1.0
2009	36	50 (18)	47 (17)	3 (1)[c]	0.001-8.0	0.06	0.25
2010	34	32 (11)	68 (23)	0 (0)	0.001-1.0	0.125	0.5
2011	37	46 (17)	51 (19)	3 (1)[c]	0.008-8.0	0.125	0.5
Total	427	25 (105)	71 (305)	4 (17)	0.001-32.0	0.25	1.0

a: Interpretative criteria for MIC classification were those described by the CLSI (CLSI 2009).

b: The MICs of 320 *N. gonorrhoeae* isolates from 2003-2008 were determined as part of this thesis. The antimicrobial susceptibility testing of 107 *N. gonorrhoeae* isolates from 2009-2011 was done at the SDCL, Regina, Canada.

c: PPNG isolates- 1/60 isolates 2003, 1/52 isolates 2005, 1/36 isolates 2009 and 1/37 isolates 2011.

Table A.2 Tetracycline susceptibility of 427 *N. gonorrhoeae* isolates from Saskatchewan (2003-2011)[a]

Year[b]	n	% Susceptible (n)	% Intermediate (n)	% Resistant (n)	MIC range (mg/L)	MIC_{50} (mg/L)	MIC_{90} (mg/L)
2003	60	22 (13)	27 (16)	51(31)	0.125-8.0	2.0	8.0
2004	59	36 (21)	22 (13)	42 (25)[c]	0.125-128.0	1.0	4.0
2005	52	15 (8)	25 (13)	60 (31)[c]	0.125-128.0	2.0	4.0
2006	55	5.4 (3)	5.4 (3)	89.1 (49)[c]	0.06-256.0	4.0	16.0
2007	53	4 (2)	9 (5)	87 (46)[c]	0.06-16.0	4.0	8.0
2008	41	5 (2)	22 (9)	73 (30)	0.03-8.0	2.0	8.0
2009	36	67 (24)	33 (12)	0	0.008-0.5	0.25	0.5
2010	34	82 (28)	18 (6)	0	0.016-1.0	0.125	1.0
2011	37	73 (27)	22 (8)	5 (2)	0.008-16.0	0.125	0.5
Total	427	30 (128)	20 (85)	50 (214)	0.008-256.0	1.0	8.0

a: Interpretative criteria for MIC classification were those described by the CLSI (CLSI 2009).

b: MIC of 320 *N. gonorrhoeae* isolates from 2003-2008 were determined were determined as part of this thesis.

The antimicrobial susceptibility testing of 107 *N. gonorrhoeae* isolates from 2009-2011 was done at the SDCL, Regina, Canada.

c: TRNG isolates- 3/59 isolates 2004, 3/52 isolates 2005, 7/55 isolates 2006, 1/53 isolates 2007.

Table A.3 Ciprofloxacin susceptibility of 427 *N. gonorrhoeae* isolates from Saskatchewan (2003-2011)[a]

Year[b]	n	% Susceptible (n)	% Intermediate (n)	% Resistant (n)	MIC range (mg/L)	MIC_{50} (mg/L)	MIC_{90} (mg/L)
2003	60	100 (60)	0 (0)	0 (0)	0.002-0.016	0.008	0.016
2004	59	100(59)	0 (0)	0 (0)	0.002-0.016	0.004	0.008
2005	52	100 (52)	0 (0)	0 (0)	0.002-0.016	0.004	0.008
2006	55	94 (52)	2 (1)	4 (2)	0.002-8.0	0.008	0.016
2007	53	96 (51)	0 (0)	4 (2)	0.004-4.0	0.008	0.008
2008	41	94 (39)	3 (1)	3 (1)	0.002-4.0	0.004	0.016
2009	36	100 (36)	0 (0)	0 (0)	0.001-0.008	0.001	0.004
2010	34	74 (25)	0 (0)	26 (9)	0.001-32.0	0.002	4.0
2011	37	86 (32)	0 (0)	14 (5)	0.001-16.0	0.002	4.0
Total	427	95 (406)	0.5 (2)	4.5 (19)	0.001-32.0	0.004	0.016

a: Interpretative criteria for MIC classification were those described by the CLSI (CLSI 2009).

b: MIC of 320 *N. gonorrhoeae* isolates from 2003-2008 were determined were determined as part of this thesis.

The antimicrobial susceptibility testing of 107 *N. gonorrhoeae* isolates from 2009-2011 was done at the SDCL, Regina, Canada.

Table A.4 Azithromycin susceptibility of 427 *N. gonorrhoeae* isolates from Saskatchewan (2003-2011)[a]

Year	n	% Susceptible (n)	% Resistant (n)	MIC range (mg/L)	MIC_{50} (mg/L)	MIC_{90} (mg/L)
2003	60	100 (60)	0 (0)	0.03-0.5	0.125	0.5
2004	59	100 (59)	2 (1)	0.016-8.0	0.125	0.5
2005	52	98 (51)	0 (0)	0.06-1.0	0.25	0.5
2006	55	100 (55)	0 (0)	0.03-1.0	0.25	0.5
2007	53	98 (52)	2 (1)	0.03-2.0	0.25	0.5
2008	41	100(41)	0 (0)	0.03-1.0	0.5	0.5
Total	320	99.5 (318)	0.5 (2)	0.016-8.0	0.25	0.5

a: Interpretative criteria for MIC classification were those described by the GISP (CDC 2010b).

Table A.5 Spectinomycin susceptibility of 427 *N. gonorrhoeae* isolates from Saskatchewan (2003-2011)[a]

Year[b]	n	% Susceptible (n)	% Intermediate (n)	% Resistant (n)	MIC range (mg/L)	MIC_{50} (mg/L)	MIC_{90} (mg/L)
2003	60	100 (60)	0 (0)	0 (0)	8.0-32.0	16.0	16.0
2004	59	81(48)	19 (11)	0 (0)	8.0-64.0	16.0	64.0
2005	52	100 (52)	0 (0)	0 (0)	8.0-32.0	16.0	16.0
2006	55	100 (55)	0 (0)	0 (0)	8.0-32.0	16.0	32.0
2007	53	94 (50)	6 (3)	0 (0)	4.0-64.0	16.0	32.0
2008	41	100 (41)	0 (0)	0 (0)	8.0-32.0	16.0	16.0
2009	36	100 (36)	0 (0)	0 (0)	4.0-16.0	8.0	16.0
2010	34	100 (34)	0 (0)	0 (0)	4.0-16.0	8.0	16.0
2011	37	100 (37)	0 (0)	0 (0)	4.0-16.0	8.0	16.0
Total	427	97 (413)	3 (14)	0 (0)	4.0-64.0	16.0	32.0

a: Interpretative criteria for MIC classification were those described by the CLSI (CLSI 2009).

b: MIC of 320 *N. gonorrhoeae* isolates from 2003-2008 were determined were determined as part of this thesis.

The antimicrobial susceptibility testing of 107 *N. gonorrhoeae* isolates from 2009-2011 was done at the SDCL, Regina, Canada.

Table A.6 Cefixime susceptibility of 427 *N. gonorrhoeae* isolates from Saskatchewan (2003-2011)[a]

Year[b]	n	MIC range (mg/L)	MIC_{50} (mg/L)	MIC_{90} (mg/L)
2003	60	0.004-0.03	0.016	0.03
2004	59	0.0005-0.06	0.008	0.03
2005	52	0.002-0.06	0.008	0.03
2006	55	0.001-0.125	0.008	0.03
2007	53	0.0005-0.016	0.008	0.016
2008	41	0.0005-0.03	0.016	0.016
2009	36	0.008	0.008	0.008
2010	34	0.008	0.008	0.008
2011	37	0.008	0.008	0.008
Total	427	0.0005-0.125	0.008	0.016

a: Break points are not defined (CLSI 2009).

b: MIC of 320 *N. gonorrhoeae* isolates from 2003-2008 were determined were determined as part of this thesis. Antimicrobial susceptibility testing of 107 *N. gonorrhoeae* isolates from 2009-2011 was done at the SDCL, Regina, Canada.

Table A.7 Cetriaxone susceptibility of 427 *N. gonorrhoeae* isolates from Saskatchewan (2003-2011)[a]

Year[b]	n	MIC range (mg/L)	MIC_{50} (mg/L)	MIC_{90} (mg/L)
2003	60	0.002-0.06	0.008	0.03
2004	59	0.00025-0.03	0.004	0.016
2005	52	0.0005-0.03	0.008	0.03
2006	55	0.001-0.06	0.016	0.03
2007	53	0.0005-0.06	0.008	0.016
2008	41	0.002-0.06	0.016	0.03
2009	36	0.001-0.016	0.004	0.008
2010	34	0.001-0.125	0.004	0.016
2011	37	0.001-0.25	0.004	0.016
Total	427	0.00025-0.25	0.008	0.03

a: Break points are not defined (CLSI 2009).

b: MIC of 320 *N. gonorrhoeae* isolates from 2003-2008 were determined were determined as part of this thesis. Antimicrobial susceptibility testing of 107 *N. gonorrhoeae* isolates from 2009-2011 was done at the SDCL, Regina, Canada.

Table A.8 Mutation patterns of PBP2 in 146 *N. gonorrhoeae* isolates susceptible to extended spectrum cephalosporins

PBP2 pattern[a]	n (%)	Ceftriaxone susceptibility groups[b]		Cefixime susceptibility groups[c]	
		1 n (%)	2 n (%)	1 n (%)	2 n (%)
IX	50 (34)	36 (29)	**14 (61)***	34(30)	**16 (67)***
I	41 (28)	**39 (32)***	2 (9)	**39 (32)***	2 (8)
XXII	35 (24)	32 (26)	3 (13)	33 (27)	2 (8.3)
II	7 (5)	7 (6)	0 (0)	7 (6)	0 (0)
XII	6 (4.0)	4 (3.3)	2 (9)	4 (3.3)	2 (8.5)
XIV	2 (1.4)	2 (2)	0 (0)	2 (2)	0 (0)
V	2 (1.4)	1 (1)	1(4)	1 (1)	1(4)
WT[d]	2 (1.4)	2 (2)	0 (0)	2 (2)	0 (0)
XXXIV	1 (0.7)	0 (0)	1(4)	0 (0)	1(4)
Total	146	123 (83)	23 (17)	122 (84)	24 (16)

a: Mutation patterns were numbered as they have been previously referenced in the literature (Whiley et al, 2007a; Ohnishi et al 2011b).
b: Ceftriaxone susceptibility groups: 1: MIC =0.0005-0.016 mg/L; 2: MIC =0.03-0.06 mg/L.
c: Cefixime susceptibility groups: 1: MIC =0.0005-0.016 mg/L; 2: MIC =0.03-0.125 mg/L.
d: WT: wild type using wild type PBP2 sequence M32091.
*: P value<0.05 is considered statistically significant.

Table A.9 PorB mutations in 146 *N. gonorrhoeae* isolates susceptible to extended spectrum cephalosporins

Pattern number	PorB mutation	n (%)	Ceftriaxone susceptibility groups[a]		Cefixime susceptibility groups[b]	
			1 n (%)	2 n (%)	1 n (%)	2 n (%)
1	WT [c]	63(43)	**60 (49)***	3 (13)	**59 (48)***	4 (17)
2	G120D; A121N	28 (19)	24 (20)	4 (17)	24 (20)	4 (17)
3	G120K; A121D	21(14)	12 (10)	**9 (39)***	12 (10)	**9 (38)***
4	G120N; A121N	11(7.5)	8 (7)	3 (13)	8 (7)	3 (13)
5	G120K; A121N	6 (4)	5 (4)	1 (4)	4 (3)	2 (8)
6	G120D; A121D	5 (3.5)	4 (3)	1(4)	5 (4)	0 (0)
7	A121S; N122K	5 (3.5)	4 (3)	1(4)	4 (3)	1 (4)
8	A121G	2 (1.4)	2 (2)	0 (0)	2 (2)	0 (0)
9	G120D	2 (1.4)	2 (2)	0 (0)	2 (2)	0 (0)
10	G120K	1 (0.7)	1(0.8)	0 (0)	1(0.8)	0 (0)
11	G120K; A121G	1 (0.7)	1 (0.8)	0 (0)	1 (0.8)	0 (0)
12	G120R; A121D	1 (0.7)	0 (0)	1(4)	0 (0)	1(4)
	Total	146	123 (83)	23 (17)	122 (84)	24 (16)

a: Ceftriaxone susceptibility groups: See Table 3.11.
b: Cefixime susceptibility groups: See Table 3.11.
c: WT: wild type using *porB* sequence M21289 (Carbonetti et al 1987).

Table A.10 *mtrR* mutations in 146 *N. gonorrhoeae* isolates susceptible to extended spectrum cephalosporins

Pattern number	MtrR mutation	n (%)	Ceftriaxone susceptibility groups[a]		Cefixime susceptibility groups[b]	
			1 n (%)	2 n (%)	1 n (%)	2 n (%)
1	WT[c]	37 (25)	**37 (30)***	0	**36 (30)***	1 (4)
2	G45D	55 (38)	42 (34)	13 (57)	43 (35)	12 (50)
3	A-;G45D	25 (17)	23 (19)	2 (9)	22 (18)	3 (12)
4	A-;H105Y	10 (7)	5 (4)	**5 (22)***	5 (4)	**5 (21)***
5	Truncated MtrR [e]	4 (3)	4 (3)	0 (0)	4 (3)	0 (0)
6	A39T (4)	4 (3)	2 (1.5)	2 (9)	2 (2)	2 (8)
7	T86A;H105Y	3 (2)	3 (2.5)	0 (0)	3 (2.5)	0 (0)
8	A40D;D79N; T86A;H105Y	3 (2)	3 (2.5)	0 (0)	3 (2.5)	0 (0)
9	D79N;T86A;H105Y	2 (1.4)	2 (1.5)	0 (0)	2 (2)	0 (0)
10	A39T;H105Y	1 (0.7)	1(0.8)	0 (0)	1 (0.8)	0 (0)
11	A-	1(0.7)	0 (0)	1 (4.5)	0 (0)	1 (4)
12	TT+	1(0.7)	1(0.8)	0 (0)	1 (0.8)	0 (0)
	Total	146	123 (83)	23 (17)	122 (84)	24 (16)

a: Ceftriaxone susceptibility groups: 1: See Table 3.11.
b: Cefixime susceptibility groups: 2: See Table 3.11.
c: WT: wild type using *mtrR* sequence Z25796 (Pan & Spratt 1994).
e: MtrR coding sequence truncated at amino acid position 136.

APPENDIX-II

Bacterial transformation

Transformation experiments to assess the role of *penA* mutations in imparting reduced susceptibility to extended spectrum cephalosporins and penicillin were carried out as described by Sarubbi et al (1974) and Ropp et al (2002). Bacterial strains used in transformations studies were FA19, WHO K, Ng 9, Ng 22 and Ng 34 (Table 2.1).

*pen*A genes were amplified with primers penF and penR (Ohnishi et al 2011b; Table 2.3) from strains FA19, WHO K, Ng 9, Ng 22 and Ng 34. The PCR mixtures were incubated for 2 min at 96°C, followed by 30 cycles of 10 s at 96°C, 10 s at 65°C, and 2 min at 72°C (Ohnishi et al 2011b). All amplicons were analyzed by agarose gel electrophoresis and purified as described in Section 2.3.2. Amplicons were transformed individually into strain FA19, as described previously (Sarubbi et al 1974; Ropp et al 2002). The transformation protocol was standardized using *penA* with mosaic *penA* pattern X, amplified from reference strain WHO K. FA19 was transformed with *penA* patterns IX, XXII and mosaic pattern XXXIV, amplified from strains Ng 9 Ng 22 and Ng 34, respectively.

Piliated colonies (Sarubbi et al 1974) of *N. gonorrhoeae* recipient strain FA19 were passaged on a fresh GCMBK plate with an inoculation loop and were grown for 12 to 16 h at 35°C in a humidified 5% CO_2 atmosphere. All cells were scrapped from these plates with an inoculation loop and were resuspended in prewarmed gonococcal broth with 1% Kellogg's supplement, 20 mM bicarbonate and 10 mM $MgCl_2$. *N. gonorrhoeae* cells were diluted to an optical density of 0.18 (at 560 nm). 20µl (~ 10 µg DNA) of purified PCR product was added to 0.9 ml cell suspension and the mixture was incubated for 5 hours at 37°C in a CO_2 (Ropp et al 2002). An aliquot (10 µl) was then inoculated onto GCMBK plates containing cefixime (0.004-0.125 mg/L) and penicillin (0.004-0.125 mg/L) in two-fold dilutions. Plates were incubated for 20-24 hours at 35^{o}C in a humidified 5% CO_2 atmosphere. Cell suspensions of the untransformed parental strain *N. gonorrhoeae* FA19 (cefixime MIC= 0.002-0.004 mg/L) were also plated on the same antibiotic series as the negative control. Untransformed strains Ng 9, WHO K, Ng 22 and Ng 34 suspensions were plated as positive controls for *penA* patterns IX, X, XXII and XXXIV, respectively. Colonies growing on cefixime and penicillin containing GCMBK plates and not

which did show growth of FA19, were subcultured on GCMBK plates without antibiotics. Single isolated colonies were subcultured three times on GCMBK plates containing cefixime (0.016 mg/L) to ensure the purity of the culture.

The antimicrobial susceptibility profiles of the generated isogenic *N. gonorrhoeae* strains were determined in duplicate using the agar dilution assays (CLSI 2009) and compared to those of wild type FA19. Recombinants showing 3-4 fold increase in the cefixime MIC values were compared to wild type FA19 and were stored to verify the transformant genotype through DNA sequencing. DNA sequencing of FA19 and FA19 transformants of *penA* pattern IX, X, XXII and XXXIV was performed as described in Section 2.5.3 previously using primers penA_SF1, penA_SF2, penA_SF3, penA_SR1, penA_SR2, penA_SR3 and penA_SR4 (Table 2.3) .

Results

Transformation with *penA* pattern XXXIV from strain Ng 34 resulted in three fold (0.004 to 0.03 mg/L) increase in cefixime MICs of the recipient strain FA19 (Table A2.1). A fourfold (0.004 to 0.06 mg/L) increase in cefixime MICs of FA19 was observed when transformed with *penA* pattern X from WHO strain K. Transformations with *penA* mosaic *penA* patterns IX and XXII from strain Ng 9 and Ng 22 did not change the cefixime MICs of FA19.

DNA sequencing of FA19 and FA19 transformants of *penA* pattern IX, X, XXII and XXXIV was done at using primers penA_SF1, penA_SF2, penA_SF3, penA_SR1, penA_SR2, penA_SR3 and penA_SR4 (Table 2.3). The sequencing reactions were done using Applied Biosystems 3730x1 DNA Analyzer (Plant Biotechnology Institute, National Research Council of Canada, Saskatoon, SK). The sequencing reactions were done twice but I could not align the whole *penA* from the FA19 and FA19 transformants because of the poor quality of the sequences. This work will be done in future since increase in the 3 to 4 fold increase in MICs of recipient strains observed after transforming mosaic *penA* patterns in recipient strain indicates that whole or partial *penA* has been inserted in *penA* of FA19.

Table A2.1 Increase in cefixime MIC values of recipient wild type *N. gonorrhoeae* FA19 transformed with *penA* mosaic patterns X and XXXIV but not with non-mosaic pattern IX and XXII

Strains and *penA* patterns	Cefixime MIC (mg/L)	Fold MIC increase
FA19 (wild type, recipient)	0.004	0
WHO K (X)	0.5	0
Ng 9 (IX)	0.06	0
Ng 22 (XXII)	0.03	0
Ng 34 (XXXIV)	0.125	0
FA19 + normal saline	0.004	0
FA19 + *penA* X (WHO K)	0.06	4
FA19+ IX	0.004	0
FA19+XXII	0.004	0
FA19 + *penA* XXXIV (Ng 34)	0.03	3

Appendix-III

Sidharath Dev Curriculum Vitae

Dev, Sidharath, D.V.M., M.V.Sc, Ph.D. Candidate
Assistant Professor, Department of Veterinary Public Health,
Dr. G.C. Negi, College of Veterinary and Animal Sciences,
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Language Competency:

- English: Reading, writing and speaking
- Hindi: Reading, writing and speaking

Education:

- Ph.D. candidate: Department of Microbiology & Immunology, University of Saskatchewan (U of Sask), Saskatoon, Canada (September 2007–August 2011). Research topic: Molecular mechanism of antimicrobial resistance and population dynamics of *Neisseria gonorrhoeae* in Saskatchewan (2003-2011). (Supervisor- Dr. Jo-Anne R. Dillon)
- Master of Veterinary Science (MVSc.): Graduate study in Veterinary Public Health, G. B. Pant University of Agriculture and Technology (GBPUA&T), Pantnagar, Uttrakhand, India (01/2000-03/2002) Research topic: Sero-epidemiology of animal and human brucellosis. (Supervisor- Dr. D.C. Thapliyal).
- Bachelor of Veterinary Sciences and Animal Husbandry (DVM): Himachal Pradesh Agriculture University (HPAU), Palampur, Kangra (H.P.), 176062; India (10/1994-11/1999).

Employment & Experience

- Junior Research Fellow in the Uttar Pradesh Council of Agriculture Research funded project "Surveillance of zoonotic diseases: Sero-epidemiology of brucellosis in animals & man in western and hill districts of UP" at GBPUA&T, Pantnagar, Uttrakhand, India (10/2000-4/2001).
    1. Determination of prevalence of zoonotic disease brucellosis in western and hill districts of Uttar Pradesh, India in man and animals.
    2. Evaluation of outer membrane proteins of *Brucella abortus* strain S-99 as vaccine candidate for brucellosis.
- Research Fellow in the National Agriculture Technology Project "Weather based animal disease forecasts" at GBPUA&T, Pantnagar, Uttrakhand, India (3/2002- 7/2003).
    1. Collection and analysis of meteorological and animal disease data from 12 districts of Uttrakhand, India to develop disease forecast models.

- Veterinary Officer in Department of Animal Husbandry, Shimla, Himachal Pradesh, India, (7/2003-12/2006).

    1. Officer in charge for 4 Veterinary hospitals and 23 Veterinary dispensaries.
    2. Prevention and cure of diseases/infections in livestock through vaccination and treatment.
    3. Organization of animal health and farmer awareness camps.

- Assistant Professor, Department of Veterinary Public Health, College of Veterinary and Animal Sciences Dr. G.C. Negi, College of Veterinary and Animal Sciences, HPAU, Palampur, Kangra (H.P.), 176062; India (12/2006 onwards).

    1. Teaching

        a. DVM courses of Veterinary Public Health and Epidemiology.
        b. Graduate level courses in Food borne infections and poisoning and zoonoses.
        c. Developed graduate level course on Food Borne Infections and Poisoning.

    2. Research
        a. Microbiological analysis of drinking water sources of district Kangra, Himachal Pradesh, India.
        b. Characterization of *Lactobacillous* spp used as probiotic in poultry ration.
        c. Investigation of rickettsial disease outbreaks in the western Himalayan region of India.
        d. Determination of efficacy of Lugol's iodine for intrauterine therapy in clinical endometritis cows.
    3. Extension

        a. Five presentations as invited speaker on topics milk hygiene, zoonosis and good animal husbandry practices to farmers.

- Research Associate with Dr. Jo-Anne R. Dillon, Department of Microbiology & Immunology, U of Sask, Saskatoon, Canada from May 1 to August 31, 2009.

    1. Worked on cell division proteins of *Neisseria gonorrhoeae* and interaction of *FtsZ* with other cell division proteins.

Awards and Honors:

- Undergraduate HPAU scholarship for Bachelor of Veterinary Sciences and Animal Husbandry/D.V.M program (1994-1999).
- Distinction in Masters in Veterinary Sciences, GBPUA&T, Pantnagar, Uttrakhand, India (2000-2002)
- Best poster award. *In: Proceeding of XVth Annual convention and National Symposium of Indian Association of Veterinary Public Health Specialists on "Recent Approaches in Veterinary Immunology and Biotechnology for Animal Health and Production".* February 26-28, 2009. Department of Veterinary Microbiology and Department of Animal Biotechnology, Chaudhary Charan Singh Haryana Agriculture University Hisar-125004, India.

- College of Graduate Studies and Research, U of Sask Scholarship (2009-2011).
- Nominated for Vanier Graduate Scholarship by U of Sask (2010-2011).
- College of Medicine Graduate Student Scholarship, U of Sask (2011-2012).
- Nominated for best graduate student award of Department of Microbiology and Immunology, U of Sask for 2011-2012.

Supervisory Experience (from 2006):

Undergraduate classes from 2006 to present:

VPH-311, 2006-2009, HPAU, India
VPH-321, 2006-2009, HPAU, India
VPH-411, 2006-2009, HPAU, India
MCIM 390, Fall 2010, Lab, U of Sask

Individual or group supervision from 2006 to present:

- Graduate students (MVSc) (1)

Memberships:

- Indian Society of Veterinary Public Health Specialists.
- Society of Immunology and Immuno-pathology.

Publications:

- Books/Manuals:

  1. Thakur, S.D. and Panda, A.K. (2008). Practical Manual on Environmental Hygiene. Department of Veterinary Public Health, HPAU, Himachal Pradesh, India-176062

- Research Papers *[Papers derived from the PhD study are indicated in bold (n=2)]*

  1. **Thakur, SD, Levett PN, Horsman GB, Dillon JR. 2013. Molecular epidemiology of *Neisseria gonorrhoeae* in Saskatchewan, Canada: association of NG-MAST strain type with antibiotic susceptibility and temporal and geographic parameters. *Sex. Trans. Infect.* (Submitted).**
  2. Sood P, **Thakur SD**, Kumar S , Dogra V, Vashishta N K , Singh MM, Gandhotra V. K. 2013. Lugol's Iodine: validation of relatively higher concentration for intrauterine use in clinical endometritis cows and effect of certain storage conditions on *in vitro* antibacterial activity. *Ind. J. Ani. Reprod.* (in press).
  3. **Vidovic S, Thakur SD, Horsman GB, Anvari V, Dillon JR. 2012. Longitudinal analysis of the evolution and dissemination of *Neisseria gonorrhoeae* strains (Saskatchewan, Canada 2005-2008) reveals three major circulating strains and convergent evolution of ciprofloxacin and azithromycin resistance. *J. Clin. Inf. Dis.* 50: 3823-30.**
  4. **Tahkur SD**, Panda AK. 2012. Laboratory investigartion of drinking water sources of Kangra, Himachal Pradesh. *J. Comm. Dis.* 44: 103-108.

5. Katoch S, **Thakur SD**, Sharma KS. 2012. Biological performance of broiler birds on varied mineral density diets supplemented with isolated direct fed microbial. *Wld. Poul. Sci. J.* (Submitted).
6. Cybulska P, **Thakur SD**, Foster BC, Scott IM, Leduc RI, Arnason JT, Dillon JR. 2011. Extracts of Canadian First Nations medicinal plants, used as natural products, inhibit *Neisseria gonorrhoeae* isolates with different antibiotic resistance profiles. *Sex. Trans. Dis.* 38: 667-71.
7. **Thakur SD**, Vaid RK, Thapliyal DC. 2007. Comparison of the immune responses induced by *Brucella abortus* S-19 vaccine and *B. abortus* S-99 outer-membrane proteins. *J. Immunol. Immunopathol*. 4: 45-49.
8. **Thakur SD**, Thapliyal DC. 2004. Seroprevalence of animal and human brucellosis in Kumaon and adjoining parts of Uttar Pradesh with comparison of serological tests. *Ind. J. Ani. Sci.* 74: 932-935.
9. **Thakur SD**, Thapliyal DC. 2002. Seroprevalence of brucellosis in man. *J. Comm. Dis.* 34: 106.
10. **Thakur SD**, Thapliyal DC. 2002. Seroprevalence of brucellosis in various animal species. *Vets India*. 25-29. (May issue).

Manuscripts in preparation: [Papers derived from the PhD study are indicated in bold (n=3)]

1. **Thakur SD, Levett P, Horsman G, Dillon JR. 2013. Molecular Epidemiology of *Neisseria gonorrhoeae* in Saskatchewan, Canada: association of NG-MAST strain type with antibiotic susceptibility and temporal and geographic parameters. (In preparation).**
2. **Thakur SD, Levett P, Horsman G, Dillon JR. 2013. Trends in antimicrobial resistance of *Neisseria gonorrhoeae* isolates from Saskatchewan: Need to make more effective use of antimicrobial agents. (In preparation).**
3. **Thakur SD, Starnino S, Levett P, Horsman G, Dillon JR. 2013. Molecular characterization of genes (*penA, penB, porB, mtrR*) associated with ceftriaxone resistance in fully susceptible *Neisseria gonorrhoeae* isolates from Saskatchewan, Canada (2003-2008). (In preparation).**
4. Araya P, Borthagaray G, **Thakur SD**, Galarza P, Llop A, Payares D, Sanabaria OM, Trigoso ME, Dillon JR. 2013. MIC creep to ceftriaxone and low levels of resistance to azithromycin in 7 countries from South America and the Caribbean. *Sex. Trans. Dis.* (In preparation).
5. Dillon JR, **Thakur SD**, Parti, RS. 2013. An overview of antimicrobial resistance in *Neisseria gonorrhoeae* in Latin America and the Caribbean countries from 1999-2011. (In preparation).
6. Ruddock PS, **Thakur SD**, Arnason JT, Durst T, Dillon JR. 2013. Antimicrobial activity of binary or ternary combinations of the phytochemicals silibinin and dillapiol with penicillin and tetracycline against *Neisseria gonorrhoeae*. (In preparation).

Meeting presentations, published abstracts/posters: [Papers derived from the PhD study are indicated in bold (n=6)]

1. **Thakur SD**, Thapliyal DC. 2002. Field efficacy of serological tests used in the diagnosis of brucellosis. *In: Proceeding of First Annual conference of Indian Association of Veterinary Public Health Specialists.* GBPUA&T, Pantnagar, Uttrakhand, India. November 28-29, 2002. pp. 36.
2. **Thakur, SD**, Thapliyal DC. 2002. *Comparison of the immune response generated by B. abortus S-19 vaccine and outer membrane proteins of B. abortus S-99. In: Proceeding of First Annual conference of Indian Association of Veterinary Public Health Specialists*. GBPUA&T, Pantnagar, Uttrakhand, India. November 28-29, 2002. pp. 39.
3. **Thakur SD**, Shrivastava S, Thapliyal DC, Garg SK. 2002. Seroprevalence of brucellosis and PPR in goats. *In: Strength, Challenges and Opportunities in Small Ruminanats Diseases in New Millennium*. Vth National Seminar. Indian Society for Sheep and Goat Production and Utilization. December 30-31, 2002, Jaipur.
4. Thapliyal DC and Thakur SD. 2003. Livestock diseases in Himalayan region: a public health perspective. *In: Livestock Production Systems for Sustainable Food Security and Livelihoods in Mountain Areas*. GBPUA&T, Pantnagar, Uttrakhand, India. December 30-31, 2002.
5. Maansi, **Thakur SD**. 2003. *In: Proceeding of Second Annual conference of Indian Association of Veterinary Public Health Specialists and National Symposium on Challenges in emerging WTO Scenario in Milk, Meat and Poultry Industry.* Department of Veterinary Public Health, Nagpur Veterinary College, Nagpur, India. September 20-22, 2003. pp. 124
6. Vaid RK, **Thakur SD**, Thapliyal DC. 2005. The in-vitro sensetivity of *Brucella abortus* S-99 to chemotherapeutic agents in general and cefotaxime in particular. *In: Proceeding of XII Annual conference of IAAVR and National Symposium on Newer Concepts and Challenges in Veterinary science and Animal Husbandry*. College of Veterinary and Animal Sciences, Bikaner. 101-102.
7. Panda AK, **Thakur SD**, Katoch RC. 2007. Rabies: Control Strategies for Himalayan States of the Indian Subcontinent. *In: 2nd International Symposium on Infectious Diseases and Health Sciences.* Department of Veterinary Pathobiology, Faculty of Veterinary Medicine and Animal Sciences, University of Peradeniya, Sri Lanka. July 26-27, 2007. 8-9.
8. Panda AK, **Thakur SD**, Garg SR, Sharma, Alok. 2007. Prevalence of the *Listeria monocytogenes* in foods of animal origin: A potential public health threat. *In: 2nd International Symposium on Infectious Diseases and Health Sciences.* Department of Veterinary Pathobiology, Faculty of Veterinary Medicine and Animal Sciences, University of Peradeniya, Sri Lanka. July 26-27, 2007. p 8-9.
9. Panda AK, **Thakur SD**. 2007. Bacteriological contaminants of foods of animal origin. *In: Proceeding of 6th Annual conference of Indian Association of Veterinary Public Health Specialists and National Symposium on horizons of Veterinary Public Health in augmenting Veterinary, Medical and Environmental Health.*. Department of Veterinary Public Health, Ananad Agricultural University, Anand-388001 Gujarat-India. November 28-29, 2002. p. 36.
10. Panda AK, **Thakur SD**, Poonam. 2008. Bacteriological Evaluation of Cow Urine

used in Organic Agricultural Practices. *In: International Symposium and VII Annual Conference of Indian Association of Veterinary Public Health Specialists on " Food Safety, Quality Assurance and Global Trade: Concerns and Strategies"* Department of Veterinary Public Health, College of Veterinary and Animal Sciences, GBPUA&T, Pantnagar, Uttrakhand, India.. November 7-9, 2008. p.139.

11. Chahota Rajesh, Sharma Mandeep, Mittra S, **Thakur SD**. 2009. Molecular epidemiological investigation of a flea borne rickettsial outbreak in the western Himalayan region based on GLTA and Omp B genes. *In: Proceeding of XVth Annual convention and National Symposium of Indian Association of Veterinary Public Health Specialists on "Recent Approaches in Veterinary Immunology and Biotechnology for Animal Health and Production".* February 26-28, 2009. Department of Veterinary Microbiolgy and Department of Animal Biotechnology, Chaudhary Charan Singh Haryana Agriculture University Hisar-125004, India.
12. **Thakur SD, Nagle E, Levett P, Horsman GB, Dillon JR. 2010. Isolates of *Neisseria gonorrhoeae* from Saskatchewan are susceptible to most antibiotics except tetracycline. *In*: *CACMID's 78th Annual Conference*. 6-8 May, 2010. Edmonton, Canada.**
13. **Thakur SD, Horsman GB, Dillon JR. 2010. Antimicrobial susceptibility of *Neisseria gonorrhoeae* isolates from Saskatchewan: time to think about regional treatment guidelines? *In: 2nd Annual RAPID Meeting*. October 20, 2010. Saskatoon, SK, Canada.**
14. **Thakur SD, Nagle E, Levett PN, Horsman GB, Liao M, Dillon JR. 2011. Emerging molecular mutations of reduced susceptibility to third-generation cephalosporins in *Neisseria gonorrhoeae* isolates from Saskatchewan, Canada. *In: 19th biennial conference of International Society for Sexually Transmitted Diseases and Research (ISSTDR)*. July 10-14, Quebec City, Canada.**
15. **Vidovic S, Thakur SD, Horsman GB, Dillon JR. 2011. Molecular Epidemiology of *Neisseria gonorrhoeae* from Saskatchewan. *In:51st Interscience Conference on Antimicrobial Agents and Chemotherapy.* September 17-20, 2011.Chicago, USA. ICAAC Final Program, 2011. pp103.**
16. **Thakur SD, Liao M, Nagle E, Levett PN, Horsman GB, Dillon JR. 2011. Identification of a *Neisseria gonorrhoeae* population susceptible to antibiotics traditionally used to treat gonococcal infections: will regional control strategies be relevant?** In: ***12th International Union against Sexually Transmitted Infections (IUSTI)*. November 2-5, 2011, New Delhi, India. Abstract book; O14: 55.**
17. Katoch S, **Thakur SD**, Sharma KS. 2012. Biological performance of broiler birds on varied mineral density diets supplemented with isolated direct fed microbial. 6th International Poultry Conference 2012, Egypt. 26-29, March 2012. (Accepted).
18. Araya P, Borthagaray G, **Thakur SD**, Galarza P, Llop A, Payares D, Sanabaria OM, Trigoso ME, Dillon JR. 2013. MIC creep to ceftriaxone and low levels of resistance to azithromycin in 7 countries from South America and the Caribbean. *In: 20th ISSTDR Conference/14th IUSTI World Congres*s. July 14-17, 2013.Vienna, Austria. A232.
19. **Thakur SD, Levett PN, Horsman GB, Dillon JR. 2013. Association of *Neisseria gonorrhoeae* NG-MAST strain types with specific mutation pattern combinations of *penA, mtrR* and *porB*. *In: 20th ISSTDR Conference/14th IUSTI***

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Other publications review articles, popular articles and lead presentations at National/International Conferences

1. **Thakur SD**, Vaid RK, Panda AK, Saini Y. 2012. Marine mammal brucellosis: a new dimension to an old zoonosis. *Curr. Sci*. 103: 902-910.
2. Panda AK, **Thakur SD**, Katoch RC. 2008. Rabies: control strategies for Himalayan states of the Indian subcontinent. *J. Commun. Dis*. 40: 169-75.
3. **Thakur SD**, Vaid RK, Barua S. 2005. Brucellosis in water buffaloes. Infectious Diseases of Domestic Animals and zoonoses in India. Ed. Tandon, V. and Dhawan, B.N. *In: Proc. Nat. Acad. Sci. India*. 75 (B)-Special issue.pp.71-84.
4. Vaid RK, **Thakur SD**, Barua S. 2005. *Brucella* diagnosis by PCR. *J. Immunol. Immunopathol*. 6: 1–8.
5. **Thakur SD**, Kumar R. 2003. Antimicrobial components of milk. *Ind. Dairyman*. 55: 61-67.
6. **Thakur SD**, Vaid RK, Kumar Mahesh. 2003. Equine Brucellosis: a review. *Centaur.* 19: 46-52
7. **Thakur SD**, Thapliyal DC, Kumar Mahesh. 2003. Current status of serodiagnosis of brucellosis. *J. Immunol. Immunopathol*. 5: 12–26.
8. **Thakur SD**, Kumar Rajesh, Thapliyal DC. 2002. Human brucellosis: Review of an under diagnosed animal transmitted disease. *J. Comm. Dis*. 34: 287.
9. **Thakur SD**, Thapliyal DC. 2002. Brucella vaccines and vaccination: a review. *Ind. J. Comp. Microbiol. Immunol. Infect. Dis*. 23: 101-110.
10. **Thakur SD**, Shrivastava S. 2001. Geographical information system: contribution to public health. *Pant. Veterinarian*. 7: 41.